**Universidade do Minho**
Escola de Ciencias da Saúde

Vânia Rita de Faria Cardoso

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# Impact of differential Toll-like receptor recognition of *Mycobacterium tuberculosis* strains on the adaptive immune response

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Trabalho Efetuado sob a orientação da
**Doutora Margarida Saraiva**
**e da**
**Doutora Lúcia Moreira Teixeira**

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*"The real voyage of discovery consists not in seeking new landscapes but in having new eyes."*

Marcel Proust

Dedico este trabalho à minha Família e ao Barreto

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## ABSTRACT

*Mycobacterium tuberculosis* (Mtb) infection, remains one of the major worldwide threats, affecting about one third of the human population. The vaccine Bacillus Calmette-Guérin (BCG), although effective against childhood disseminated tuberculosis (TB), has variable and limited efficacy against adulthood pulmonary TB. Co-infection with the human immunodeficiency virus (HIV) and the emergence of multidrug resistant strains of Mtb has further complicated the TB control and eradication. It is becoming increasingly recognized that the Mtb complex (MTBC) is more genetically diverse than previously believed. In our laboratory, we have been interested in the study of the differential host-pathogen interactions among Mtb strains, particularly those from the Beijing family. Among this Mtb lineage, we found that strain 02-171, is recognized by both toll-like receptor 2 (TLR2) and TLR4, resulting in a distinct outcome of infection *in vitro* and *in vivo*.

In the first part of this work we investigated the impact of TLR4 triggering on T cell responses during a primary infection by 02-171 Mtb strain via the aerosol route. We found that TLR4 triggering increased the frequency of lung CD4+ T cells, which appears to be associated with enhanced T cell recruitment. Furthermore, TLR4 activation did not impact interferon (IFN)-γ response neither nitric oxide synthase 2 (*Nos2)* expression. In accordance, TLR4 triggering did not impact bacterial growth after aerosol 02-171 infection. Interestingly, TLR4 absence increased lung interleukin (IL)-17 CD4+ T cell responses. Overall, our data suggest no apparent function for TLR4 triggering during a primary infection with a low dose of 02-171 Mtb strain inoculated via the aerosol route.

In the second part of this work, we focused on the role of TLR4 activation by 02-171 Mtb strain during a recall response after BCG vaccination. We found that BCG vaccination is effective at controlling the progression of infection and lung pathology in 02-171 infection. This finding does not support the idea that Beijing strains are resistant to protection evoked by BCG vaccination. In vaccinated mice, we observed an overall anticipation of protective responses, particularly the presence of multifunctional and granulocyte macrophage colony-stimulating factor (GM-CSF)-producing CD4+ T cells. Furthermore, while wild-type (WT) vaccinated mice upregulated IL-17 responses, in TLR4 deficient mice such induction was not observed, without impairment in BCG-induced protection. Overall, TLR4 triggering seems to be not essential for BCG-mediated immunity against 02-171 infection. Understanding, the mechanisms that dictate the development of adaptive immunity will certainly contribute for the identification of T cell subsets and other immune factors important for protection against Mtb infection, as well as, novel correlates of protection, particularly relevant to evaluate the effectiveness of new TB vaccine candidates.

## RESUMO

A infeção por *Mycobacterium tuberculosis* (Mtb) constitui um grave problema em todo o mundo, afetando cerca de um terço da população humana. A vacinação com o Bacilo Calmette-Guérin (BCG), embora eficaz contra tuberculose (TB) disseminada na infância, tem uma eficácia variável e limitada contra a TB pulmonar em idade adulta. A co-infeção com o vírus da imunodeficiência humana (HIV) e o aparecimento de estirpes multirresistentes de Mtb contribuiu para o deficiente controlo e erradicação da TB no mundo.

Mtb é geneticamente mais heterogéneo do que inicialmente se acreditava. O nosso laboratório interessa-se pelo estudo da heterogeneidade das interações patogénio-hospedeiro com estirpes de Mtb, nomeadamente as da família Beijing. Recentemente, publicámos que a estirpe 02-171 desta linhagem ativa o recetor do tipo Toll 2 (TLR2), como também o TLR4, com consequências na resposta do hospedeiro *in vitro* e *in vivo*.

Na primeira parte do trabalho nós investigamos o papel da ativação do TLR4 no desenvolvimento de respostas T durante uma infeção primária pela estirpe 02-171. Nós observamos que a ativação do TLR4 induz um aumento na frequência de células T CD4+ do pulmão, o que parece estar associado com um aumento do recrutamento dessas mesmas células. Apesar da ativação do TLR4 não ter afetado a resposta do interferão (IFN)-γ nem a expressão da óxido nítrico sintase 2 (*Nos2)*, a ausência deste recetor causou o aumento da resposta IL-17 pelas células T CD4+ no pulmão. Em suma, os dados obtidos neste trabalho sugerem que a ativação do TLR4 durante uma infeção primária por 02-171 não confere proteção contra a infeção.

Na segunda parte do trabalho focámo-nos no papel da ativação do TLR4 pela estirpe 02-171 durante a resposta de memória após a vacinação com BCG. Observamos que a vacinação é eficaz no controlo da progressão da infeção e inflamação pulmonar induzida pela estirpe 02-171, não suportando a ideia de que estirpes Beijing são resistentes à proteção conferida pela vacinação com BCG. Em ratinhos vacinados, observamos uma antecipação global da resposta protetora, em particular a presença de células T CD4+ multifuncionais e produtoras de GM-CSF. Além disso, nos ratinhos *wild-type* (WT) vacinados observamos um aumento da resposta IL-17, enquanto em ratinhos vacinados deficientes para TLR4 o aumento desta resposta induzida pela vacinação não foi observada, sem qualquer prejuízo para a proteção induzida pela imunização por BCG. Em suma, a ativação do TLR4 não parece ser essencial para a imunidade mediada por BCG após a infeção com 02-171. Perceber os mecanismos que determinam o desenvolvimento da imunidade adaptativa contribuirá para a identificação de subpopulações de células T e outros fatores imunológicos importantes para a proteção contra TB, bem como, para a identificação de novos biomarcadores de proteção, particularmente relevantes para a avaliação da eficácia de novas vacinas.

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## LIST OF ABBREVIATIONS

**ANOVA** Analysis of variance

**APC** Antigen presenting cell

**AraLAM** Arabinofuranosyl lipoarabinomannan

**BCG** Bacillus Calmette-Guérin

**BMDM** Bone marrow-derived macrophages

**BrdU** Bromodeoxyuridine

**BSA** Bovine serum albumin

**CCR** C-C chemokine receptor

**CD** Cluster of differentiation

**cDMEM** Complete Dulbecco's Modified Eagle Medium

**CFU** Colony forming units

**$CO_2$** Carbon dioxide

**CpG** Cytosine-phosphate-guanine

**CXCL** C-X-C motif chemokine

**CXCR** CXC chemokine receptor

**DAPI** 49,6-diamino-2-phenylindole hydrochloride

**DC** Dendritic cell

**DC-SIGN** Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin

**DEPC** Diethylpyrocarbonate

**DMEM** Dulbecco's Modified Eagle Medium

**ELISA** Enzyme-linked immunosorbent assay

**FACS** Fluorescence-activated cell sorting

**FBS** Fetal bovine serum

**G-CSF** Granulocyte-colony stimulating factor

**GLA** Glucopyranosyl Lipid Adjuvant

**GM-CSF** Granulocyte macrophage colony stimulating factor

**H&E** Hematoxylin and eosin

**$H_2O_2$** Hydrogen peroxide

**HIV** Human immunodeficiency virus

**HPRT** Hypoxanthine phosphoribosyltransferase

**IFN** Interferon

**IL** Interleukin

**iNOS** Inducible nitric oxide synthase

**IP** Intraperitoneal

**IRF** IFN regulatory factor

**KLRG1** Killer-cell lectin like receptor G1

**LAM** Lipoarabinomannan

**LM** Lipomannan

**LN** Lymph node

**LSP** Large sequence polymorphisms

**Ly6G** Lymphocyte antigen 6G

**Man-LAM** Mannose-capped lipoarabinomannan

**MAPK** Mitogen-activated protein kinase

**MCP** Monocyte chemoattractant protein

**MDR** Multi-drug resistant

**MHC** Major histocompatibility complex

**MPL** Monophosphoryl lipid A

**Mtb** *Mycobacterium tuberculosis*

**MTBC** *Mycobacterium tuberculosis* complex

**MVA85A** Modified Vaccinia Ankara virus expressing antigen 85A

**MyD88** Myeloid differentiation factor 88

**NF-κB** Nuclear factor kappa-B

**$NH_4Cl$** Ammonium chloride

**NK** Natural Killer

**NKG2D** NK group 2, member D

**NKp46** NK cell p46-related protein

**NLR** Nucleotide-binding oligomerization domain-like receptor

**NO** Nitric oxide

**NOD** Nucleotide-binding oligomerization domain

**NOS2** Nitric oxide synthase 2

**OADC** Oleic acid/ albumin/ dextrose/catalase

**ON** Overnight

**PAMP** Pathogen associated molecular pattern

**PB** Proskauer Beck

**PBS** Phosphate-buffered saline

**PD-1** Programmed cell death 1

**PIM** Phosphatidyl-myo-inositol mannoside

**PMA** Phorbol myristate acetate

**PRR** Pattern recognition receptor

**R** Receptor

**Rag** Recombination-activating gene

**RFLP** Restriction fragment length polymorphism

**RIG** Retinoic acid-induced gene

**RLR** Retinoic acid-induced gene-I-like receptor

**RNI** Reactive nitrogen intermediate

**ROI** Reactive oxygen intermediate

**ROR-γt** Retinoic acid receptor related orphan receptor-γt

**RT** Room temperature

**RT-PCR** Real-time polymerase chain reaction

**SCID** Severe combined immunodeficiency

**SE** Stable oil-in-water emulsion

**SEM** Standard error of the mean

**STAT** Signaling transducer and activator of transcription

**TAP** Transporter associated with antigen processing

**TB** Tuberculosis

**T-bet** T-cell-specific T-box transcription factor

**TCR** T cell receptor

**TGF** Transforming growth factor

**Th** T helper

**TIR** Toll-interleukin 1 receptor

**TIRAP** TIR domain-containing adaptor protein

**TLR** Toll-like receptor

**TNF** Tumour necrosis factor

**TRAM** TRIF-related adaptor molecule

**Treg cell** Regulatory T cell

**TRIF** TIR domain-containing adaptor protein inducing IFN-β

**WHO** World health organization

**WT** Wild-type

**XDR** Extensively drug-resistant

# INTRODUCTION

## 1.1 Tuberculosis: a problem with major proportions

Tuberculosis (TB) is an infectious respiratory disease that essentially occurs in the lung, although it can also affect other organs (as lymph nodes (LN), meninges and bone) – extrapulmonary TB [1, 2]. Despite the increasing understanding of the disease pathogenesis, TB still has a significant impact on public health worldwide (Figure 1), being the second leading cause of death from an infectious disease, second only to the human immunodeficiency virus (HIV) [1]. In 2013, the World Health Organization (WHO) Global Tuberculosis Report estimates that there were approximately 8.6 million new TB cases and around 1.3 million TB deaths (1.0 million among HIV-negative people and 0.3 million HIV-associated TB deaths) [1]. The number of deaths resulting from TB is still incongruously high, given that most of the cases are avoidable if patients had access to primary health care for diagnosis and if the right treatment was provided and accomplished [1]. Nowadays, despite the descending trends in the incidence rates of the disease, the situation in Portugal is considered one of the most severe among Western European countries (Figure 1) [3].

TB is a highly transmittable disease, due to the fact that it is disseminated through the air [1]. When a patient with active disease sneezes, coughs or spits, the bacilli that cause the disease spread into the air and can then be inhaled by surrounding people [1]. It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb), the causative agent of most cases of TB [1, 2, 4], with only 5 to 10% of those people developing active disease with symptoms (fever, fatigue, cough with bloody sputum, night sweats and weight loss) and culturable bacilli in sputum [2, 5, 6]. In the remaining TB cases, the infected individuals develop an effective immune response that culminates in latent infection without clinical symptoms [7]. In those cases mycobacteria rests in the host inside the granuloma – a hallmark of TB disease [8, 9]. However, those individuals are susceptible to disease reactivation, which normally happens in situations associated with immunosuppression [7]. Just a small percentage of individuals remain uninfected upon exposure to Mtb possibly owing to the expression of high innate immunity [8, 10]. In the past years, TB renewed a new significance mainly due to diabetes, co-infection with HIV and the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of Mtb [2, 6, 7, 11].

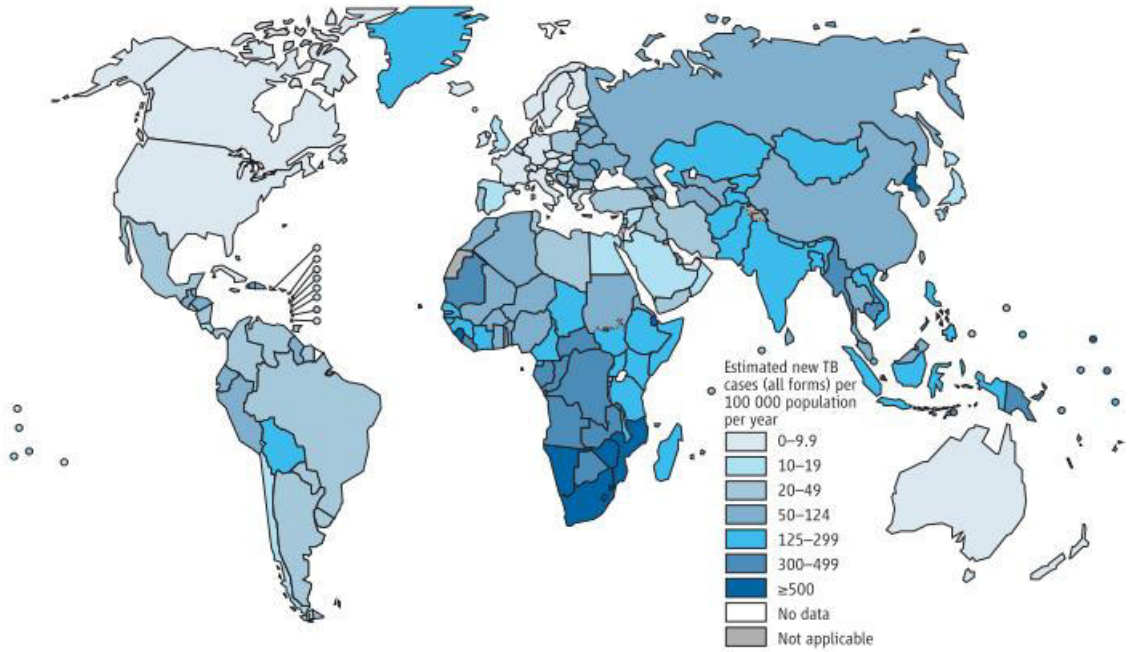


**Figure 1 –** Estimated worldwide TB incidence rates in 2012, as determined by the WHO. From [1].

## 1.2 *Mycobacterium tuberculosis*

TB is caused by a group of phylogenetically related species collectively denominated as Mtb complex (MTBC) [9, 12]. Besides Mtb, other *Mycobacterium* species can cause disease like *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium microti, Mycobacterium bovis* and *Mycobacterium avium* in immunocompromised individuals [9]. Mtb is considered a Gram-positive and an acid-fast bacteria, characterized by slow growth, dormancy and genetic variability among Mtb strains [5, 12]. Mtb is a facultative intracellular bacteria that grows inside phagocytic cells, particularly macrophages and monocytes. One of the most strictly characteristics of this pathogen is the complex cell wall, rich in lipids. The cell wall core comprises peptidoglycan bound to arabinogalactan, which in turn is attached to mycolic acids. The most external layer, also denominated capsule, consists in mannose-capped lipoarabinomannan (Man-LAM), lipomannan (LM), and manno-glycoproteins [13, 14].

Genomic studies revealed that Mtb consists in 7 main lineages and 15 sublineages based on the analysis of large sequence polymorphisms (LSPs) and other phylogenetic markers [12, 15, 16]. Each lineage is linked to specific geographical regions. For instance, in Europe, the Euro-American lineage of Mtb prevails, which suggest that lineages of Mtb are more adapted to particular human populations [15].

The Beijing genotype (East-Asia lineage) has been described as one of the most successful lineages of Mtb [17, 18]. Strains are classified as belonging to the Beijing family based on their distinct spoligotype and IS*6100* restriction fragment length polymorphism (RFLP) patterns [17, 18]. Strains of this clade were firstly found in China and Mongolia, but their frequency has emerged worldwide, representing nowadays about 13% of the isolates [17, 18]. Strains of the Beijing genotype are a major concern, because of their global association with worldwide TB epidemics, high virulence and mortality, increased drug resistance, non-protective immune responses and possible inefficacy of Bacillus Calmette–Guérin (BCG) vaccination [17-21].

### 1.3 *Mycobacterium tuberculosis* recognition by Toll-like receptors

The innate immune response is initiated by the activation of germline-encoded receptors, globally designated as pattern recognition receptors (PRRs), which recognize highly conserved microbial features, in a wide range of pathogens [22, 23]. These microbial structures, known as pathogen-associated molecular patterns (PAMPs), are essential biomolecules of the microorganisms, that therefore do not suffer changes easily [22]. During infection, several host cell receptors are involved in the pathogen recognition [22]. For example, in the case of Mtb, the mannose receptor, complement receptors, dectin-1, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), mincle, nucleotide-binding oligomerization domain (NOD-) like receptor (NLR), retinoic acid-induced gene (RIG)-I-like receptor (RLR), Fc receptors, scavenger receptors and toll-like receptors (TLRs) are involved in pathogen recognition by innate immune cells [8, 13, 24, 25]. From here on, we focus on TLRs, the scope of this thesis.

TLRs are expressed on several immune cells, including macrophages, dendritic cells (DCs), some T cell subsets, B cells and also non-immune cells such as epithelial cells and fibroblasts [26] [22]. To date, 12 TLRs have been characterized in mice, while the human TLR family comprises 10 known members [23, 24]. TLRs differ from each other essentially in ligand specificity, expression patterns and signaling pathways triggered downstream TLR activation [22]. Moreover, TLRs can be expressed either extra- or intracellularly. In that sense, TLR1, -2, -4, -5 and -6 are expressed on the cell surface and therefore recognize mainly pathogen membrane structures [23, 24]. In contrast, TLR3, -7, -8 and -9 are localized in intracellular organelles and recognize mostly nucleic acids [23, 24]. The TLR triggering by PAMPs activates intracellular signaling cascades that induce the expression of genes involved in antimicrobicidal mechanisms [23, 24]. Upon ligand binding the interaction among TLRs and adaptor proteins is initiated. Myeloid differentiation factor 88 (MyD88)

adaptor molecule is critical for all TLR signaling with exception of TLR3 (Figure 2). TLR3 signals exclusively via toll-interleukin 1 receptor (TIR) domain-containing adaptor protein inducing interferon (IFN)-β (TRIF) adaptor molecule, while TLR4 utilizes both MyD88 and TRIF (Figure 2). In the plasma membrane, TLR4 interacts with TIR domain-containing adaptor protein (TIRAP) and MyD88 triggering signaling cascades that culminates in the activation of nuclear factor kappa-B (NF-κB) or in the phosphorylation of mitogen-activated protein kinase (MAPK), leading to the production of proinflammatory cytokines and chemokines that intervene in the immune response (Figure 2). After internalization, TLR4 form a complex with TRIF-related adaptor molecule (TRAM) and TRIF (Figure 2). In addition to NF-κB and MAPK activation, the TRIF-dependent pathway leads to the activation of IFN regulatory factors (IRFs) with consequent transcription of type I IFN (Figure 2) [23, 24].

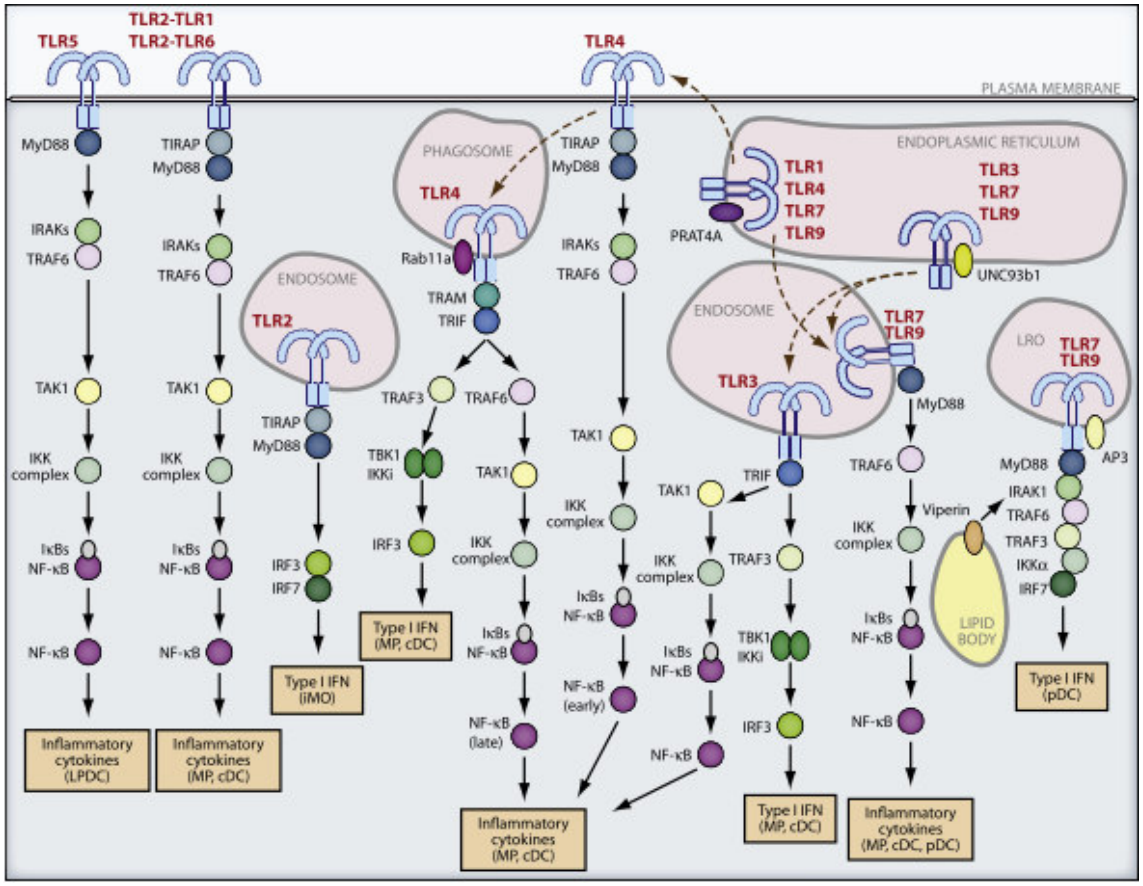


**Figure 2 –** TLR signaling pathway. From [23].

TLR2 in association either with TLR1 or TLR6, TLR4 and TLR9 have been described as mediating *in vitro* and *in vivo* recognition of Mtb [8, 27-34]. TLR2 is involved in the recognition of 38-kDa

protein, 19-kD glycoprotein, LAM, LM, arabinofuranosyl LAM (AraLAM), phosphatidyl-myo-inositol mannoside (PIM), triacylated (TRL2/TLR1) and dyacylated (TLR2/TLR6) lipoproteins of Mtb. TLR4 also recognizes PIM and heat-shock protein 60/65, while TLR9 is activated by unmethylated cytosine-phosphate-guanine (CpG) motifs in mycobacterial DNA [8, 13, 30].

As anteriorly referred, TLR2 is known to recognize several mycobacteria ligands, being accepted as the main TLR for the *in vitro* activation upon Mtb infection [8]. *In vitro* studies of TLR activation showed that TLR2 triggering by Mtb ligands causes the inhibition of macrophage antigen presentation via major histocompatibility complex (MHC) class II and also blocks the response of macrophages to IFN-γ, which suggest that TLR2 signaling could negatively impact macrophage functions [8]. Additionally, TLR2 and TLR9 are important receptors for driving interleukin (IL)-12 production in response to Mtb [30].

Data from our laboratory showed that in a panel of Mtb strains, including the reference Mtb strain H37Rv, and strains from the Beijing lineage, most of the Mtb strains were recognized by TLR2 by bone marrow-derived macrophages (BMDM) [34]. However, we also found that Mtb Beijing strain 02-171 was also recognized by TLR4 [34]. This Mtb strain induced a different profile of cytokines in BMDM, with high pro- and anti-inflammatory responses [34]. Interestingly, only 02-171-infected BMDM produced IFN-β, a type I IFN [34].

*In vivo* studies showing a fatal infection of mice deficient for MyD88, evidence the role for TLR activation in the control of Mtb infection [35, 36]. Nevertheless, the involvement of individual TLR signaling for the protection against Mtb infection remains to be elucidated. *In vivo* experimental Mtb infection using mice deficient for specific TLRs suggests a differential role of TLRs in Mtb infection. Mice deficient for TLR9 display an increased susceptibility to high dose of Mtb infection than wild-type (WT) animals [30]. Also upon a high dose of Mtb infection TLR2 defective mice showed a reduced bacterial clearance compared to WT mice [28, 33]. However, in response to a low dose of Mtb infection, TLR2 and -9 deficient mice presented similar protection compared to WT mice [30, 33]. Furthermore, TLR2 triggering has a key role on the regulation of p19 (*Il-23a*) expression in response to Mtb and therefore impact the maintenance of T helper 17 (Th17) cells in the lung of Mtb-infected animals [27].

Regarding the role of TLR4 triggering during Mtb there are inconsistent results. Some studies showed that TLR4-defective C3H/HeJ mice were as resistant to aerosol Mtb infection as C3HHeN control mice [32, 33]. In contrast, other reports showed that TLR4 mutant mice were more susceptible to Mtb infection when compared to WT mice [29, 31]. Recently, our group showed that

TLR4 deficient mice were more susceptible to an intranasal infection by a TLR4-activating Mtb strain, while upon infection by a TLR2-activating Mtb strain TLR4 deficient mice were as resistant as WT mice, supporting the involvement of TLR4 during a protective response to at least some strains of Mtb [34].

Human genetic variations have been reported within the TLR genes, which might affect their functionality and increase TB susceptibility. Indeed, some human TLR4 polymorphisms, mainly TLR4 Asp299Gly and Thr399Ile polymorphisms, have been associated with TB susceptibility in Asian Indian and in a cohort of HIV-infected Tanzanian patients [37, 38]. However, in Southeastern Iran, Gambia, South Indian and Southeastern Chinese population the same association was not found [39-43]. Different polymorphisms in TLR2 in cohorts from Turkey, Vietnam, Pakistan, Southeast Iran and Korea associated with increased susceptibility to TB [44-47], whereas such association was not observed in cohorts from Southeastern China and South India [41, 43, 48]. Finally, TLR9 polymorphisms have also been shown to associate with TB in cohorts from Mexican, China, Indonesia and Vietnam [49-51]. However, the same association was not found in Southeastern Iran and India [39, 41]. These discrepant data might be explained on the basis of a dynamic host-pathogen genetic and pathogen phenotypic interplay. Indeed, further studies are needed to clarify the specific contribution of TLRs for the outcome of Mtb infection.

TLR triggering can also play a role during adaptive immunity [52, 53]. In the absence of T cell receptor (TCR) signaling, murine Th1 effector T cells can be activated by TLR2 ligands with consequent IFN-γ production. In those conditions, TLR2 agonists can also stimulate proliferation and survival of Th1 cells [53, 54]. Moreover, TLRs induce T cell differentiation through the activation antigen presenting cells (APCs) that in response to TLR binding produce polarizing cytokines, as IL-12 and IL-23, and chemokines, necessary for cell recruitment to the site of infection [53]. Thus, T cell activation can be induced either through T cell/APC interactions or directly via TLR activation in the absence of APCs. TLR2 deficiency by compromising p19 (*IL-23a*) expression limits Th17 cell responses to Mtb infection [27]. Memory T cells are also responsive to TLR ligands. In this case, TLR triggering could help in T cell survival and in the mounting of a fast memory response [53]. Furthermore, TLR agonists can temporally suppress regulatory T cell (Treg) function, allowing the activation of pathogen-specific T cells [53].

## 1.4 Innate immune response to *Mycobacterium tuberculosis*

Upon aerosol exposure, Mtb spreads into the lungs, firstly infecting the alveolar resident macrophages [5, 6]. After that, other cells take part in this process. APCs, such DCs and macrophages, are the main innate immune cells involved in protection against TB [6, 55]. Following Mtb recognition, TLR triggering in APCs promotes the phagocytic process and activates their microbicidal mechanisms in order to eliminate or reduce Mtb growth [8]. Upon phagocytosis and inside the phagolysosome (formed by phagosome fusion with lysosomes), Mtb is eradicated through several mechanisms, such as decrease of the phagosomal pH, production of reactive oxygen intermediates (ROIs) such as hydrogen peroxide ($H_2O_2$) and reactive nitrogen intermediates (RNIs) like nitric oxide (NO), and release of hydrolytic enzymes or other antimicrobicidal components into the phagosome [56]. However, Mtb can resist to macrophage microbicidal mechanisms and survive due to modulation of the host immune response, for instance preventing the phagolysosome fusion [6, 57]. Upon activation by TLRs, and in the presence of IFN-γ and tumor necrosis factor (TNF), activated macrophages produce NO via inducible nitric oxide synthase (iNOS) enzyme using L-arginine and molecular oxygen as substrates [5, 56, 58]. In murine models of infection, *Nos2* (the gene encoding for iNOS) deficient (NOS2-/-) mice have an impaired clearance of Mtb infection compared to WT-infected mice, corroborating the protective role of NO against Mtb infection in mice [5, 56, 58]. However, whether NO production is relevant to human macrophage microbicidal mechanisms remains unclear [6].

Despite the important function of macrophages in eliminating Mtb infection, these cells are poor activators of naïve T cells [59]. In contrast, DCs are the main APCs able to sense, uptake, process and present mycobacterial antigens [8]. Upon recognition, immature DCs phagocyte Mtb or Mtb-infected apoptotic cells at the site of infection. Upon maturation, DCs migrate to draining LN to present mycobacterium antigens coupled with MHC molecules to naïve T lymphocytes initiating an antigen-specific adaptive immune response [4, 55]. During this maturation process, costimulatory molecules as cluster of differentiation (CD)40, CD80, CD86 and MHC-II molecules are upregulated and polarizing cytokines, as IL-12, IL-23, IL-1β, TNF and IL-6 involved in T cell differentiation, and chemokines are secreted (Figure 3) [4, 22, 55, 60, 61]. All these processes are controlled by PPR activation on DCs.

Despite the central role of DCs and macrophages in innate immunity against Mtb, other innate immune cells as neutrophils, natural killer (NK) cells and γδ T cells are also involved in Mtb challenge [5, 56]. For instance, after Mtb infection neutrophils are rapidly recruited to the site of

infection, where they phagocytize bacteria [62]. However, the role of neutrophils is still unclear. Despite conflicting, studies where neutrophils were depleted and mice infected with Mtb suggest that these cells might contribute to early defense against Mtb infection. Nevertheless, in the chronic phase of the disease neutrophil accumulation is most likely to contribute to pathology [62].

NK cells are granular lymphocytes with both cytotoxicity and cytokine-producing effector functions [63]. NK cells are able to lysis Mtb-infected cells, such monocytes, through the cytotoxicity receptors as NK cell p46-related protein (NKp46) and NK group 2, member D (NKG2D) [64, 65]. In addition, NK cells also produce IFN-γ, which contributes for the initiation of macrophage microbicidal mechanisms, resulting in early resistance to many infections, such as *Listeria monocytogenes* [66]. In a mouse model with intact T cell function, antibody depletion of NK cells during *in vivo* Mtb infection, does not cause an impairment in Mtb control [67]. In contrast, studies in severe combined immunodeficiency (SCID) mice have suggested a putative protective role for NK cells in the immune response to Mtb [66]. Indeed, IFN-γ secreted by NK cells does not seem to be essential for normal control of Mtb infection, but this response may become important in situations in which T cell function is compromised, such as in HIV patients or SCID mice [66].

γδ T cells, are a subtype of T cells that classically belongs to the innate arm of the immune system [68]. γδ T cells, differ from classical αβ T cells, in the range of antigens that they can recognize, as non-peptide antigens, independently of MHC-II [68]. γδ T lymphocytes are able to efficiently kill extracellular and intracellular Mtb by releasing the cytotoxic molecule granulysin [68]. γδ T cells are also able to secrete IFN-γ [68]. In addition to IFN-γ, it has been reported that γδ T cells are the main source of IL-17 production in response to Mtb infection [69]. In the mouse model, the absence of γδ T cells results in defective granuloma formation, however, the bacterial burden in the lungs was similar to the exhibited by WT mice [70]. The precise role of these cells in Mtb infection remains unclear [68].

## 1.5 Adaptive immune response to *Mycobacterium tuberculosis*

### 1.5.1 General

In contrast to innate immunity, adaptive immunity relies on highly specialized cells that react specifically to pathogen antigens and have the ability to generate immunologic memory that leads to an enhanced and fast response to following encounters. B (humoral immunity) and T (cell mediated immunity) cells are the main effector cells of the adaptive immune arm [71].

The role of B cells during Mtb infection is still unclear. Murine models of B cell deficiency showed that after high, but not low inoculum dose of Mtb, mice were more susceptible to infection compared to control mice [4, 72, 73].

T cells play a fundamental protective role in immunity to TB [2, 4, 5, 56]. CD8+ T cells or cytotoxic T cells recognize the cognate antigen in the context of MHC-I molecules and besides cytokine production, such as IFN-γ, that activates macrophage activity, CD8+ T cells can directly eliminate Mtb-infected cells by the release of perforin, granzymes, and granulysin [56, 74]. Studies with β2-microglobulin (a component essential for MHC-I expression), transporter associated with antigen processing 1 (TAP-1) (involved in the transport of cytosolic peptides to the endoplasmic reticulum, where they bind to MHC-I molecules) and CD8α gene disrupted mice suggest a protective role of CD8+ T cells in Mtb infection [5, 56, 74].

CD4+ T cells or Th cells recognize peptides presented by MHC-II molecules and act essentially by cytokine production [56]. Since the study of CD4+ T cell response is the scope of this thesis, we now focus mainly in CD4+ T cell responses.

### 1.5.2 CD4+ T cell responses during *Mycobacterium tuberculosis* infection

T cells mainly CD4+ T cells have extreme importance in resolution of Mtb infection in human and in mice [4, 5, 56]. This fact was confirmed by the observation that individuals infected with HIV, who present low numbers of CD4+ T cells, are very susceptible to Mtb [2, 4, 75]. Also murine studies have shown, by antibody depletion of CD4+ T cells or by the use of genetically manipulated mice, that the CD4+ T cells are required for Mtb control [4, 5, 56, 76].

Upon Mtb infection, DCs phagocyte Mtb at the site of infection. Mature DCs migrate to the LN to present processed mycobacterium antigens to naïve T lymphocytes [4]. After TCR engagement and depending of cytokine milieu and co-stimulation provided by APCs, naïve CD4+ T cells are activated, proliferate and become committed to one of several lineages of Th cells, including Th1, Th17 and Treg cells among others (Figure 3) [4, 77, 78]. Effector Th cells migrate to the lungs in response to specific inflammatory mediators and develop an immune response that further promotes activation of macrophage microbicidal mechanisms [4].

Following Mtb challenge, the induction of T cell polarizing cytokines is dependent of PRRs that are expressed and activated in APCs [4]. In the absence of IFN-γ and after Mtb-induced TLR2-dependent DCs stimulation, IL-23 is higher expressed than IL-12 [79], whereas in the presence of IFN-γ Mtb-infected DCs secrete both IL-12 and IL-23 which potentiate both Th1 and Th17

polarization [79, 80]. *In vitro* and after Mtb infection both TLR2 and TLR9 activation induce the production of IL-12p40 by DCs [30, 80]. IL-12p40 subunit is shared by IL-12 and IL-23, key innate cytokines involved in Th1 and Th17 responses. Consequently the innate stimulus that leads to the production of these cytokines has an important impact in the type of Th response and has to be different and highly regulated [80]. Recently, we reported that certain Beijing strains of Mtb preferentially activate TLR2, whereas others also activate TLR4 resulting in a distinct cytokine pattern *in vitro* and *in vivo* [34]. Thus, it is possible that distinct Mtb strains by differentially stimulating DCs, lead to secretion of distinct polarizing cytokines which may impact the Th cell response generated. Indeed, the work developed in this thesis focus on the impact of TLR4 triggering by a TLR4-activating Mtb strain on the development of adaptive immunity.

Each T cell lineage is characterized by their cytokine production profile, function and different patterns of cell surface molecules (Figure 3) [33, 77, 78]. The signaling pathways triggered in CD4+ T cells by polarizing cytokines leads to the activation of signaling transducer and activator of transcription (STAT) proteins which are involved in induction and binding of the main transcription factors to genes that are under their influence [77, 81]. The hallmark cytokine of Th1 cells is IFN-γ, but these cells also produce IL-2, TNF and lymphotoxin (Figure 3) [77, 78, 82]. The main cytokine involved in Th1 polarization is IL-12 (Figure 3) [77, 78, 81]. IL-12 is a heterodimer formed by two subunits namely IL-12p35 and IL-12p40 that signal through the IL-12 receptor (IL-12R) composed by IL12Rβ1 and IL12Rβ2 chains [83]. Mtb infected-DCs secrete IL-12 that potentiates IFN-γ production by those cells [84]. T box expressed in T cells (T-bet) is the master transcription factor associated with IFN-γ production and Th1 differentiation (Figure 3) [77, 78, 81]. After TCR engagement, the binding of IFN-γ to IFN-γR triggers the activation of STAT1 and T-bet [77, 78]. Following, T-bet further induces the production of IFN-γ and IL-12R, which potentiates the action of IL-12 and subsequently the Th1 expansion and maintenance (Figure 3) [77, 78, 85]. STAT4 is another protein that is involved in Th1 differentiation and intervenes in IL-12 signaling pathway (Figure 3) [77, 78, 85].

In Mtb infection, Th1 cells are fundamental for protective immunity [2, 4, 5, 56]. The main functions of IFN-γ secretion in Mtb infection are to enhance macrophage phagocytosis and increase the induction of NO and ROIs, which are mechanisms involved in pathogen clearance [5, 56]. Humans with defects in the production or signaling of IL-12 and IFN-γ cytokines are highly susceptible to TB [4, 86]. Furthermore, the importance of IL-12 and IFN-γ was demonstrated in mice deficient in those molecules that succumbed early in response to Mtb infection [56]. Studies

revealed that IL-12p40 deficient mice are more susceptible to Mtb infection than IL-12p35 knockout mice, suggesting a protective role for IL-12p40 [2]. Indeed, upon Mtb infection IL-12p40 homodimers are involved in DC migration and T cell activation [2, 84]. Additionally, the arrival and accumulation of IFN-γ-producing T cells in the lungs is temporally associated with the stop of bacterial growth [4, 87, 88]. However, a recent report using a model of adoptive transfer of Mtb specific effector CD4+ T cells, propose a mycobactericidal effector function of CD4+ T cells that is independent of both IFN-γ and TNF production [89]. These findings show that, despite the fact that CD4+T cells and IFN-γ are important for Mtb control, CD4+ T cells can mediate protection by other IFN-γ-independent mechanisms during primary and secondary challenge [89, 90].

Upon Mtb infection, and in addition to Th1 cells, Th17 cells are also induced [80]. Th17 cells secrete IL-17A (IL-17), IL-17F and IL-22 as their signature cytokines (Figure 3) [80, 91]. The cytokines that drive Th17 differentiation are IL-6, IL-21 and transforming growth factor (TGF)-β in low quantities (Figure 3) [80, 91]. Others cytokines such IL-1β and TNF act as cofactors during Th17 development [80, 91]. The main transcription factor involved in Th17 polarization is retinoic acid receptor related orphan receptor-γt (ROR-γt) [80, 91]. IL-6, IL-23 and TFG-β intervene through activation of STAT3 pathway, which in turn induces ROR-γt and IL-23R expression that allows Th17 differentiation and maintenance (Figure 3) [80, 91, 92]. IL-23 is a heterodimeric cytokine formed by IL-12p40 and IL-23p19 subunits [91]. IL-23 plays an important function not in initial differentiation but in the later maintenance of Th17 phenotype, since that IL-23R is not expressed in naïve CD4+ T cells [77, 80, 91, 92]. During intracellular bacterial infections as Mtb infection the function of IL-17 is not well understood [71, 80, 93]. Recent reports propose that IL-17 confers protective immunity against some intracellular pathogens like *Salmonella enterica* and *Listeria monocytogenes* [76, 80]. In low dose of Mtb infection, the lack of IL-17 and IL-23 does not have a significant impact in the capacity of mice to limit Mtb infection. Although following a high dose challenge, IL-17 is required to the resolution of Mtb infection [80]. Th17 cells have an important role in induction of tissue inflammation and in development of immunopathology [80, 91, 93]. IL-17 is a pro-inflammatory cytokine that induces the expression of several cytokines like IL-6, IL-8, IL-1β, TNF, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage–colony stimulating factor (GM-CSF); chemokines, as monocyte chemoattractant protein-1 (MCP-1), C-X-C motif chemokines 1 (CXCL1), CXCL9, CXCL10 and CXCL11 and antimicrobial proteins as defensins [80, 91]. Due to the induction of these chemokines by IL-17, this cytokine has an impact in neutrophil recruitment and inflammation. Indeed, after BCG infection and in absence of IL-17

the number of neutrophils recruited upon Mtb infection decreases as compared with WT mice [80]. Thus, upon Mtb infection, the IL-23/Th17 axis might be preponderant for granuloma organization, because it promotes chemokine secretion that potentiates an early neutrophil recruitment. Furthermore, the accumulation of neutrophils can potentiate IL-12 production and stimulate Th1 differentiation [80]. However, if excessive IL-17 production occurs, continuous neutrophil recruitment leads to an exacerbate immune response and tissue damage [80, 94]. This exacerbated immune response can be inhibited by many regulators such as IFN-γ, IL-27 and IL-10 whose signaling pathways inhibit Th17 differentiation preventing immunopathology [91, 92]. Thus, Th17 cells may have contrasting functions during Mtb infection either in fighting infection or in promoting tissue damage [80].

In sum, a fine balance between Th1 and Th17 responses is needed to stop bacterial growth and restrict immunopathology [80]. In this sense, Treg cells downregulate the immune response generated to avoid exacerbated pathology by secreting anti-inflammatory cytokines such as IL-10 and TGF-β (Figure 3). However, during Mtb infection Treg cells seem to play a detrimental role since they prevent pathogen clearance by suppressing protective CD4+ T cell responses [95].

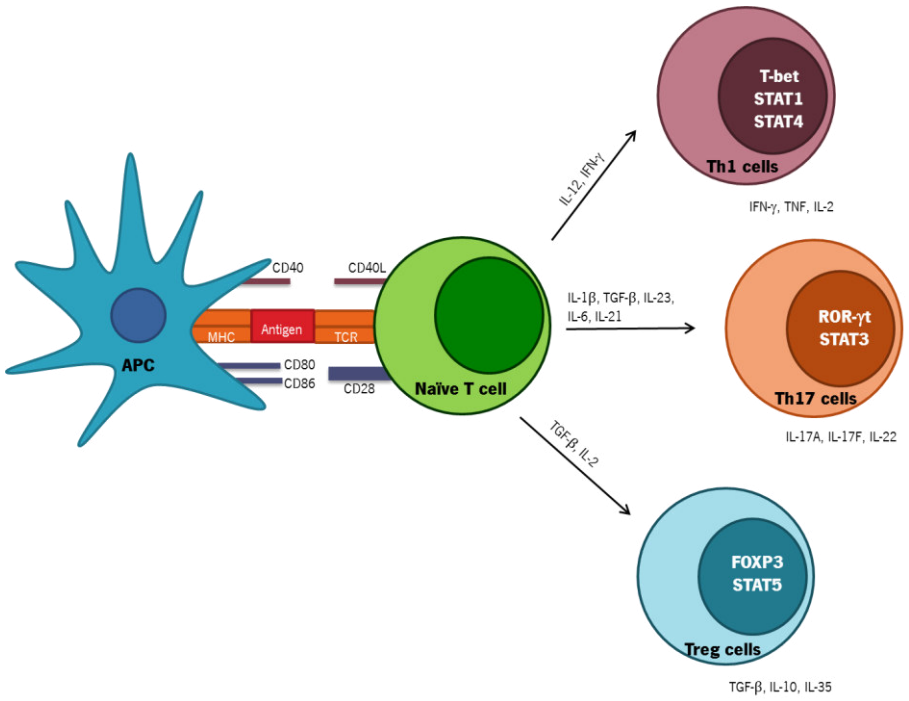


**Figure 3 – Subsets of effector Th cells.** Depending on the cytokine milieu present at the time of antigenic stimulation, naïve CD4+ T cells can differentiate into various subsets of Th cells (Th1, Th17, Treg and others). Specific transcription factors have been identified as master regulators of each subset. Differentiated Th cells are characterized by the secretion of a combination of effector cytokines. Adapted from [77, 78, 85, 91, 95].

One particular feature of TB is the slow induction of the adaptive immunity, which allows an uncontrolled growth of mycobacteria and the establishment of a chronic infection, which is detrimental for the outcome of infection [4]. The mechanisms responsible for the delayed T cell priming remain elusive, but some assumptions have been proposed [4]. It was demonstrated that dissemination of Mtb-infected DCs to LN is essential to priming T cell responses [4, 55, 96, 97]. One hypothesis is that Mtb suppresses APC function, thereby delaying the dissemination of antigens from the lungs to the LN. The slow bacterial growth of Mtb may also contribute for the delay in the initiation of adaptive immunity, since a long time is needed to reach an adequate antigen concentration to stimulate the immune system [4, 10, 98]. Effective levels of adaptive immune responses are only achieved after 18-20 days post-infection and consequently the bacterial growth and dissemination become controlled [4]. Between 2 to 6 weeks post-infection, the influx of lymphocytes and activated macrophages to the local of infection results in granuloma formation [4]. This structure consists in infected phagocytes delimited by activated giant cells and epitheloid macrophages and an external layer of lymphocytes and fibrosis. The granuloma main function is to encircle Mtb in immune cells and impede dissemination and transmission of infection into the rest of the uninfected lung [99, 100].

The dynamics and characteristics of the T cell response induced upon Mtb infection needs further investigation in order to understand which T cell subpopulations are important for protection and how do they differentiate. This knowledge will certainly contribute for the development of new and more effective vaccines against TB.

## 1.6 TB prevention and treatment

### 1.6.1 BCG vaccination

BCG is currently the only available vaccine against TB. BCG is a live strain of *Mycobacterium bovis* developed in 1921 by Calmette and Guérin through consequent passages of a virulent strain of *M. bovis* that suffered attenuation [101, 102]. This vaccine is able to interact with different components of the innate and adaptive immune systems [101, 102]. Currently, BCG is administrated intradermally soon after birth. However, the efficiency of this vaccine is variable, ranging from 0 to 80%, and it only prevents the dissemination of disease in childhood, not avoiding the development of pulmonary TB in adulthood [101, 102]. The observed variation in BCG efficiency can be due to several factors like: early exposure to environmental mycobacteria,

differences in BCG sub-strains or in subsequent Mtb strains infecting the individual, inadequate stimulation of immune system by BCG, age of administration and host genetic variation [101, 102]. In addition, the protection conferred by BCG is limited and has the duration of 10 to 20 years [101, 102]. However, a second immunization with BCG or other mycobacterial preparations may not be a good approach because repeated exposure of mice with previous latent Mtb infection, to mycobacterium antigens was shown to induce an exacerbated immune response leading to severe lung damage [94].

### 1.6.2 The immune response underlying BCG vaccination

Although BCG has been used for many years, being one of the most widely used vaccines in the world, our knowledge on the cellular and molecular mechanisms involved in the protection conferred by BCG is still limited [101].

Achievement of BCG mediated protection, requires antigen-specific effector T cells to be recruited quickly to the site of infection and to activate the infected phagocytes [4, 103]. Furthermore, these cells should be able to persist within the infection site [104, 105].

As discussed before, IFN-γ-producing CD4+ T cells are essential for Mtb control and for the host survival [106, 107]. For this reason, IFN-γ-producing T cells have been widely used as a correlate of protection upon Mtb infection [108, 109]. However, the level of IFN-γ production does not necessarily associate with vaccine mediated protection [108, 110], suggesting that other immune factors are also important for T cell-mediated protection. Indeed, it has been demonstrated that BCG vaccinated IFN-γ-deficient mice, although limited, exhibit protection against a subsequent Mtb aerosol infection, in a mechanism dependent of CD4+ T cells [90, 110].

Th17 cells have also been associated with vaccine-induced protection by anticipating Th1 arrival to the infected lung [83]. As explained before, Th17 cells increase the expression levels of chemokines responsible for attracting T cells, such as CXCL9, 10, and 11, which in turn enhance the recruitment of IFN-γ-producing T cells to the site of infection [103]. Nevertheless, repeated exposure of mice, with a stabilized Mtb-infection, to BCG vaccination leads to an uncontrolled IL-17 cellular response that induce tissue damage related with increased neutrophil recruitment to the lung [94].

Clearly, CD4+ effector T cells comprise several T cell subsets, going from early activated T cells only producing IL-2, single cytokine secretors such as IFN-γ- or IL-17-producing T cells, to CD4+ T cells that might have several effector functions by the simultaneous secretion of various immune

factors such as IFN-γ, TNF and IL-2 [4]. The presence of these multifunctional T cells has been associated with protection in mouse models of *Leishmania major* infection [111]. Given that IFN-γ by itself was not associated with vaccine-induced protection, it is expected that CD4+ T cells secreting multiple cytokines might play an important function in the control of Mtb infection [108]. However, the protective role of these cells in TB still unclear [112]. Mtb-infected mice primed with BCG and boosted with Modified Vaccinia Ankara virus expressing antigen 85A (MVA85A) showed a higher control of bacterial burden in the lungs that was associated with the presence of multifunctional T cells producing IFN-γ, TNF and IL-2 compared with BCG alone [108]. Also, studies of BCG vaccination with infants demonstrated the presence of T cells that secrete several combinations of IFN-γ, TNF and IL-2 [113]. However, another study reported that adults with active Mtb infection expressed higher multifunctional CD4+ T cells than individuals with latent disease [114]. This observation suggests that multifunctional CD4+ T cells might associate with active disease, instead of protective immunity. Moreover, it seems that the presence of CD4+ T cells secreting multiple cytokines correlates with bacterial load, as indicated by the reduction of multifunctional T cells in TB subjects after the end of TB therapy [114].

It has been reported that BCG immunization is less protective against Beijing Mtb strains when compared with H37Rv infection, a reference laboratory strain [19, 21]. In a study performed by Ordway *et al.* until day 30 post-aerosol infection, mice infected with Beijing strains were equally protected comparative to H37Rv-infected ones. However, at day 60 post-infection the protection conferred by BCG was lost in Beijing-infected mice. This observation suggests that BCG protection against the Beijing strains tested was only transient and fails in long term-protection. This raises the question that BCG immunization or other BCG-like vaccines could be unsuccessful in regions of the world with high prevalence of Beijing genotype strains [20]. In contrast, other study demonstrated no strain-specific differences in BCG-induced protection [115]. Also, in humans, some studies tried to correlate BCG inefficacy with Beijing endemic areas, while others failed in demonstrating such association [19]. In our laboratory, in a panel of Beijing Mtb strains, we found a Mtb strain that in addition to TLR2 also triggers TLR4, with an impact for the outcome of Mtb infection. Thus, in this thesis, we investigated whether BCG vaccination is equally effective in inducing protection against this Beijing TLR4-activating Mtb strain [34]. In addition, taking into account the particular feature of this Mtb strain we want to understand the role of TLR4 triggering on the development of CD4+ T cell responses evoked by BCG vaccination.

### 1.6.3 Novel vaccination approaches

The impairment of BCG vaccination in protecting the host against TB in addition to the emergence of drug resistant strains of Mtb highlights the urgency of alternative strategies for prevention and therapy. Therefore, it is of extreme importance understand the underlying mechanisms that mediate both natural and vaccine-induced immunity [116]. Several strategies have been implemented to develop a better vaccine against TB. However most of them are based on boosting the immune response to BCG, in order to improve their effectiveness. As explained before, BCG is not an ideal vaccine and the protection conferred is not life-long, although it can prevent the most severe childhood manifestations of TB [101, 102]. Thus, the achievement of a most effective vaccine against TB may require the development of a booster vaccine [101, 102]. For instance, BCG vaccination early in life followed by an adjuvant to increase the strength and the durability of an effective immune response [117].

The most advanced of these improved vaccines is MVA85A that enhanced the protective efficacy of BCG in animal models by delivering the immunodominant antigen 85A as a booster using a replication-deficient poxvirus [118]. Recently, the phase IIb of clinical trials was completed but no significant protection against Mtb was obtained after MVA85A administration in infants [119].

Adjuvants can be added to vaccines such as in Hepatitis A virus vaccine in order to enhance effective T cell responses. PAMPs, specifically those binding the TLRs, are the basis of many adjuvants [11]. Therefore, the new generation of vaccines often integrates agonists of TLRs to enhance T helper cell responses [120]. The glycolipid monophosphoryl lipid A (MPL ®), which activates TLR4, was the first TLR ligand used as adjuvant in an approved human vaccine (the Hepatitis B vaccine Fendrix ®) [120]. M72 consisting of Mtb72F polyprotein formulated with the adjuvant AS02A (a MPL® based adjuvant) has been tested as a TB candidate vaccine [117, 118]. M72 was administrated as a boost of BCG in the cynomolgus monkey model demonstrating superior protection than that afforded by using BCG alone [121]. Furthermore this vaccine was well tolerated by healthy adult subjects in a phase I clinical trial [118]. Another study showed that boosting of BCG with the recombinant fusion protein ID93, adjuvanted with the TLR4 agonist Glucopyranosyl Lipid Adjuvant (GLA), formulated in a stable oil-in-water emulsion (SE) (ID93+GLA-SE), protects mice and guinea pigs against Mtb compared to BCG alone [122]. ID93+GLA-SE is presently under Phase I clinical trials [123].

### 1.6.4 TB treatment

Since BCG is not effective in preventing TB, treatment with anti-TB drugs is mandatory. Generally the treatment of drug-sensitive Mtb stains can be performed with a combination of first line drugs which comprise isoniazid, rifampin, pyrazinamide and ethambutol for at least 6 months. However, if the therapy is not successful, for instance with MDR and XDR strains, a second line of anti-TB drugs such as kanamycin, amikacin, capreomycin, para-aminosalicyclic acid, cycloserine, prothionamide and thiacetazone has to be implemented for at least 2 years [124, 125].

Therefore it is clear that alternative strategies for prevention and therapy are urgently needed to tackle the TB burden in the world.

# AIMS

Previous data from our laboratory demonstrated that different Mtb strains trigger different TLRs, with impact in both *in vitro* and *in vivo* immune responses. Using *in vitro* and in *in vivo* models of infection we showed that 02-171 Mtb strain from the Beijing lineage is recognized not only by TLR2, but can also trigger TLR4. In contrast, the reference strain H37Rv mainly activates TLR2. Interestingly, we observed that TLR4 deficiency led to a higher susceptibility of mice upon intranasal infection with 02-171 Mtb strain, whereas the same did not occur in H37Rv-infected mice, thus suggesting a protective role for TLR4 triggering [34]. Given that TLR activation could influence the differentiation of T cells [52, 53], which are fundamental for protection against TB [2, 4, 5, 56], in the Chapter I of this thesis we focus on the consequences, at the adaptive immune level, of TLR4 activation during a primary mycobacterial infection by 02-171. Therefore the main goals of this chapter were to investigate:

**i)** the role of TLR4 activation on T cell priming, activation and recruitment;

**ii)** the consequence of TLR4 activation on the lung pathology and granuloma organization upon infection by 02-171 Mtb strain.

Recent studies suggest that BCG is less effective to protect mice against infection with Beijing strains comparative to the strain H37Rv [19, 21]. Given that BCG is mainly recognized by TLR2 [126], we speculated that the type of acquired immunity induced by BCG immunization may be protective against TLR2-activating Mtb strains, but defective towards a TLR4-activating Mtb strain challenge. Thus, we hypothesized that the differential TLR recognition of Mtb strains could also account for the variability of BCG protection. In this line, Chapter II focuses on the role of TLR4 activation by 02-171 Mtb strain during a recall response after BCG vaccination. The main goals of this part of the work were to evaluate:

**i)** the effectiveness of BCG vaccination in protecting WT mice against Mtb infection with 02-171 Mtb strain;

**ii)** the impact of TLR4 triggering in the effectiveness of BCG vaccination against TLR4-activating Mtb strain infection;

**iii)** how TLR4 activation during Mtb infection influences BCG-induced CD4+ T cell responses.

The knowledge of how natural and vaccine induced immunity generated in response to Mtb infection could have a huge impact on the development of new strategies of vaccination and therapy.

# MATERIAL AND METHODS

## Animals

WT C57BL/6 mice were purchased from Charles River (Barcelona, Spain). TLR4 deficient (TLR4−/−) mice were breed at the ICVS animal facility. NOS2 deficient (NOS2−/−) mice were kindly provided by Dr. Rui Appelberg, Institute for Molecular and Cell Biology (IBMC), University of Porto, Porto, Portugal. All mice were kept at the ICVS animal facility under conventional conditions, with food and water given *ad libitum*. Both male and female mice between the ages of 8 and 12 weeks were used. All procedures involving animals were carried out in accordance with the European Union Directive 86/609/EEC, and previously approved by the Portuguese National authority *Direcção Geral de Veterinária*.

## Bacterial strains and growth conditions

Mtb clinical isolate 02-171 was kindly provided by Dr. Gunilla Källenus Karolinska Institutet, Sweden and *Mycobacterium bovis* BCG Pasteur (hereafter simply referred as BCG) was originally from the Trudeau Institute Mycobacterial Collection. Bacteria were first grown at 37°C in Middlebrook 7H9 Broth (BD Bioscience) for 7–10 days and then diluted into Proskauer Beck (PB) medium supplemented with 30% glycerol and 0.05% Tween 80. At mid-log phase bacterial stocks were collected and frozen in 1 mL aliquots at -80°C.

## Experimental aerosol infection and BCG vaccination

Mice were infected via the aerosol route by using an inhalation exposure system (Glas-Col) calibrated to deliver a dose of 100 to 200 colony forming units (CFUs) of Mtb 02-171. In some experiments a lower aerosol infection dose of nearly 70 CFUs was used. The mycobacterial inoculum was prepared from the frozen stock, firstly by passing it through a 26G needle 6 times to disrupt bacterial clumps and then diluted in water (Aqua B. Braun) to the desirable concentration. Then, 10 mL of this suspension were placed into the nebulizer and the mice were exposed to the aerosol cloud for 40 minutes. The infection dose was confirmed for every experiment by plating the entire lung 3 days after the aerosol infection, as described below.

In some experiments, mice were subcutaneously vaccinated in the chest with $10^6$ CFUs of BCG and rested for 90 days before Mtb aerosol infection.

**Preparation of single cell suspensions**

At day 3 and selected time-points post-infection mice were killed by $CO_2$ asphyxiation and the organs were aseptically excised. Lungs were first perfused with cold phosphate-buffered saline (PBS) through the right ventricle of the heart until the lungs appeared white, minced and incubated for 30 minutes at 37°C with collagenase IX (0.7mg/mL, from Sigma-Aldrich). Digested lungs, LN or livers were disrupted by passage through a 40-μm-pore-size nylon cell strainer (BD Biosciences). LN cells were resuspended in complete Dulbecco's Modified Eagle Medium (cDMEM, DMEM supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 1% of HEPES, 1% L-glutamine and 1% sodium pyruvate (all from GIBCO) and used for bacterial burden determination. Liver cells were used for bacterial burden determination and lung suspensions were treated with a red blood cells lysis buffer (0.87% of $NH_4Cl$ solution and 5% of PBS in water) to remove red blood cells and resuspended in cDMEM. Lung single cell suspensions were enumerated with a Countess Automatic Cell Counter (Life Technologies).

**Bacterial Burden determination**

Bacterial burdens were determined by incubating single cell suspensions with 0.1% saponin (Sigma-Aldrich) for 10 minutes at room temperature (RT) to disrupt the cells that harbor Mtb. CFUs were determined by plating 10-fold serial dilutions of the disrupted cell suspensions in Middlebrook 7H11 (BD Biosciences) agar plates supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC) and 0.5% glycerol. BBL™ MGIT™ PANTA™ antibiotic mixture (BD Bioscience) was used to prevent contaminations. Viable mycobacteria colonies were enumerated after 3 weeks of incubation at 37°C. Values were transformed in $Log_{10}$ before plotting and statistically analyzed.

**Flow cytometry analysis of single cell suspensions**

For cell surface antigen staining antibodies against CD8-FITC (clone 5H10-1 from BioLegend); CD69-PerCP-Cy5.5 (clone H1.2F3 from BD Biosciences); CD4-APC-Cy7 (clone GK1.5 from BioLegend); CD3-PerCP-Cy5.5 (clone 145-2C11 from BioLegend); killer-cell lectin-like receptor G1 (KLRG1)-APC (clone 2F1 from eBioscience); programmed cell death 1 (PD-1)-PECy7 (RMP1-30 from BioLegend ) CD11b-PE (clone M1/70 from BioLegend); lymphocyte antigen 6G (Ly6G)-APC (clone 1A8 from BioLegend) were used. Single cell suspensions were first incubated with Fc block (BioLegend) for 10 minutes on ice and then stained in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% of FBS plus 0.01% of azide) for surface markers at 4°C for 30 minutes.

Cells were then washed with FACS buffer, resuspended in PBS containing 2% of formol and kept overnight (ON) at 4°C in the dark before analyzed.

For intracellular cytokine staining, cell suspensions were restimulated with a mixture of phorbol myristate acetate (PMA) (50ng/mL) and ionomycin calcium salt (4μg/mL) for 4 hours, in the presence of brefeldin A (10μg/mL) (all from Sigma-Aldrich). For the detection of cytokine production, antibodies against TNF-FITC (clone MP6-XT22 from BioLegend); GM-CSF-PE (clone MP1-22E9 from eBioscience); INF-γ-PE-Cy7 (clone XMG1.2 from eBioscience); IL-2-PB (clone JESG-5H4 from BioLegend) and IL-17-APC (clone TC11-18H10.1 from BioLegend) were used. Upon surface staining, cells were fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience) in the dark for 30 minutes at 4°C. Cells were washed with the Permeabilization/Wash buffer (eBioscience) and incubated with a cocktail of fluorochrome-labeled antibodies at 4°C for 30 minutes, in the dark. Finally, cells were resuspended in PBS containing 2% of formol and kept ON at 4°C in the dark.

For Bromodeoxyuridine (BrdU) staining mice were subjected to an intraperitoneal injection (IP) of 0.8 mg of BrdU (Sigma-Aldrich) 24 hours before harvest. BrdU staining was performed with APC BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, after surface staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm buffer for 20 minutes at 4°C in the dark. Cells were then washed with BD Perm/Wash buffer and resuspended in BD Cytofix/Cytoperm PLUS and incubated 10 minutes at 4°C in the dark. After washing, cells were fixed again with the BD Cytofix/Cytoperm Buffer for 5 minutes at 4°C in the dark, washed and incubated with DNAse (300μg/mL in PBS), to expose incorporated BrdU, for 1 hour at 37°C in the dark. Cells were then washed and stained with anti-BrdU antibody (APC) for 30 minutes at RT in the dark. Finally, cells were resuspended in PBS containing 2% of formol and kept ON at 4°C in the dark.

Unstained cells were used to access autofluorescence and single stained controls were performed for fluorescence compensation. Samples were acquired on a LSRII flow cytometer (BD Bioscience). All data were analyzed using FlowJo software (TreeStar). The frequency of responding cells was determined and applied to the number of cells per sample to generate the total number of specific cell populations per organ.

## RNA extraction and quantification

Total lung RNA was extracted by using TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Briefly, glycogen (20μg/μL from Roche) was added to each sample and incubated for 5 minutes at RT. After incubation, 50μL of chloroform (Sigma-Aldrich) were added and the samples were mixed and incubated on ice for 15 minutes. After centrifuging at 13000 rpm for 15 minutes at 4°C, the upper aqueous phase (containing RNA) was carefully transferred to a new tube, and mixed with an equal volume of isopropyl alcohol (Sigma-Aldrich) to precipitate the RNA. Samples were incubated ON at -20°C and then centrifuged at 13000 rpm for 15 minutes at 4°C before removing the supernatant and washing the RNA pellet with 800μL of 70% ethanol (Carlo Erba reagents). Ethanol was completely removed after centrifugation at 9000 rpm for 5 minutes and the dried RNA pellet resuspended in RNase/DNase-free water (Gibco). RNA concentration was measured (Nanodrop ND-1000 Spectrophotometer) and purity assessed through the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios.

## cDNA synthesis and RT-PCR

cDNA synthesis was performed with the RevertAid H minus kit (Fermentas life sciences) according to the manufacturers' instructions. Briefly, 1μL of OligodT, 1μL of diethylpyrocarbonate (DEPC) water, 1μL of Riboblock, 1μL of Reverter Aid, 4μL of Reaction buffer and 2μL of dNTP per sample were mixed with 10μL of RNA sample. The cDNA synthesis reaction was performed in thermocycler (Bio-Rad) at 42°C for 60 minutes following by 70°C for 15 minutes. The resultant cDNA template was used for quantification of target genes by RT-PCR analysis with SYBR green or TaqMan detection systems. Ubiquitin or hypoxanthine phosphoribosyltransferase (HPRT) were used as housekeeping genes. For SYBR Green based reactions 1μL of cDNA sample was added to 9μL of RT-PCR mix that contains 3μL of water, 1μL of forward and reverse primer and 5μL of SYBR green (Qiagen). RT-PCR was carried out in thermocycler (Bio-Rad) using the following cycling parameters: 95°C for 15 min, followed by 40 amplification cycles of 95°C for 15 seconds, 58°C for 20 seconds and 70°C for 15 seconds, and the melting curve analysis.

For TaqMan based reactions 1μL of cDNA sample was added to 9μL of RT-PCR mix that contains 3.5μL of water, 0.5μL of primers and 5μL of TaqMan Gene Expression Master Mix (Applied Biosystems). RT-PCR was carried out in thermocycler (Bio-Rad) using the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by 60°C for 1 minute.

Relative mRNA expression of the gene of interest was normalized to the levels of the housekeeping gene using the ΔCt method:

$$1.8^{(\text{Housekeeping gene mRNA expression} - \text{Target gene mRNA expression})} \times 100000$$

A control without template was done in parallel. The sequences of primers and the references of the TaqMan primer-probe sets used in RT-PCR are listed in Table I and II, respectively.

**Histology and immunohistochemistry**

The right upper lobe of the lung was inflated with 10% neutral buffered formalin and submerged during a week at 4°C in the same solution before to be embedded in paraffin.

For histological analysis sections of 3µm thickness stained by hematoxylin and eosin (H&E) were processed routinely by light microscopy. For immunofluorescence staining of iNOS, tissue sections of 3µm thickness were desparaffinized with xylene and rehydrated in steps from absolute ethanol to distilled water. Antigens were retrieved using Citrate buffer (Thermo Scientific) in a 96°C pre-warmed water bath for 30 minutes. The sections were then washed for 5 minutes with PBS solution containing 0.1% (v/v) Triton X-100 and 0.1% Tween-20. From here on, incubation periods were carried out in a humidified chamber to prevent tissue sections drying. Tissue sections were blocked for one hour at RT with 0.1% (v/v) Triton X-100, 0.1% Tween-20 and 5% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS and incubated ON at 4°C with a goat anti-mouse NOS2 antibody (M-19G, Santa Cruz) followed by an Alexa Fluor 568-conjugated polyclonal rabbit anti-goat (Invitrogen), for one hour at RT, in the dark. Finally, slides were mounted using Vectashield mounting medium containing DAPI (49,6-diamino-2-phenylindole hydrochloride) (Invitrogen), to stain nuclei.

Representative images were obtained with an Olympus BX61 microscope and were recorded with a digital camera (DP70) using the cell^P software. The immunohistochemistry images were processed using ImageJ software.

**Statistical analysis**

The results are given as means ± standard error of the mean (SEM) of at least four animals for experimental group, as indicated in the figure legends. Differences between means were analyzed by two-way Analysis of variance (ANOVA) with Bonferroni post-hoc test in Graph Pad Prism 5 software. Values were considered significant for $p \leq 0.05$ and defined as *, $p \leq 0.05$; **, $p \leq 0.01$ and ***, $p \leq 0.001$.

**Table I –** Sequences of the SYBR Green specific oligonucleotides for ubiquitin and target genes used for quantification of mRNA expression by RT-PCR.

| GENES | PRIMERS | SEQUENCE |
|---|---|---|
| **Ubiquitin** | Sense | 5'- TGGCTATTAATTATTCGGTCTGCAT -3' |
| | Anti-sense | 5'- GCAAGTGGCTAGAGTGCAGAGTAA -3' |
| **IL-17** | Sense | 5'- CTCAGACTACCTCAACCGTTCCA -3' |
| | Anti-sense | 5'- TTCCCTCCGCATTGACACA -3' |
| **TNF** | Sense | 5'-GCCACCACGCTCTTCTGTCT-3' |
| | Anti-sense | 5'-TGAGGGTCTGGGCCATAGAAC-3' |
| **IL-12b (p40)** | Sense | 5'- CAAATTACTCCGGACGGTTC -3' |
| | Anti-sense | 5'- AGAGACGCCATTCCACATGTC -3' |
| **IL-23a (p19)** | Sense | 5'- CGTATCCAGTGTGAAGATGGTTGT -3' |
| | Anti-sense | 5'- GCTCCCCTTTGAAGATGTCAGA -3' |

**Table II –** References of the TaqMan primer-probe sets for HPRT and target genes used for quantification of mRNA expression by RT-PCR.

| GENES | PRIMER-PROBE SET REFERENCE (APPLIED BIOSYSTEMS) |
|---|---|
| **IFN-γ** | Mm01168134_m1 |
| **NOS2** | Mm00440502_m1 |
| **CXCL9** | Mm00434946_m1 |
| **CXCL10** | Mm 99999072_m1 |
| **CXCL11** | Mm00444662_m1 |
| **HPRT** | Mm00446968_m1 |

# RESULTS – CHAPTER I

## IMPACT OF TLR4-TRIGGERING ON T CELL RESPONSES DURING A PRIMARY INFECTION WITH A TLR4-ACTIVATING MTB STRAIN

### 1.1 Activation of TLR4 by 02-171 Mtb strain during primary infection impacts the frequency but not the number of CD4+ T cells in the lungs

Previous data from our laboratory demonstrated that different strains of Mtb trigger different TLRs [34]. Using *in vitro* and in *in vivo* models of infection, we showed previously that 02-171 Mtb strain from the Beijing lineage besides being recognized by TLR2 also triggers TLR4, comparatively to H37Rv that preferentially activates TLR2. Importantly, this differential recognition of Mtb strains impacts both *in vitro* and *in vivo* immune responses [34]. Moreover recombination-activating gene (Rag)2 -/- mice infected via the intranasal route with Mtb strain 02-171 succumbed earlier than H37Rv-infected ones and exhibited higher bacterial loads in the lungs at day 30 post-infection, suggesting that, despite the increased virulence of 02-171 during the innate phase of the immune response, an efficient and protective T cell response was differentiated in response to Mtb 02-171 [34].

Thus, to further understand the impact of TLR4-triggering on T cell response during a primary infection, we infected WT and TLR4 -/- mice via the aerosol route with a low dose of 02-171 Mtb strain, a TLR4-activating strain.

We firstly compared the presence of CD4+ and CD8+ T lymphocytes in the lungs of WT and TLR4 -/- 02-171-infected mice by flow cytometry. As expected, the total number of CD4+ and CD8+ T cells in the lungs only increased from day 20 post-infection (Figure 1B and C) which coincides with the arrival to and accumulation of T cells in the infected lungs. Interestingly, at day 25 post-infection we observed an increased frequency of CD4+ T cells in the lungs of WT mice compared with TLR4 -/- 02-171-infected mice (Figure 1A and B). However, this difference was not translated to the total number of this T cell population (Figure 1B). Furthermore, the frequency and the total number of CD8+ T cells in the lungs were similar in both strains of mice (Figures 1A and C), indicating that TLR4 activation did not impact CD8+ T cell responses.

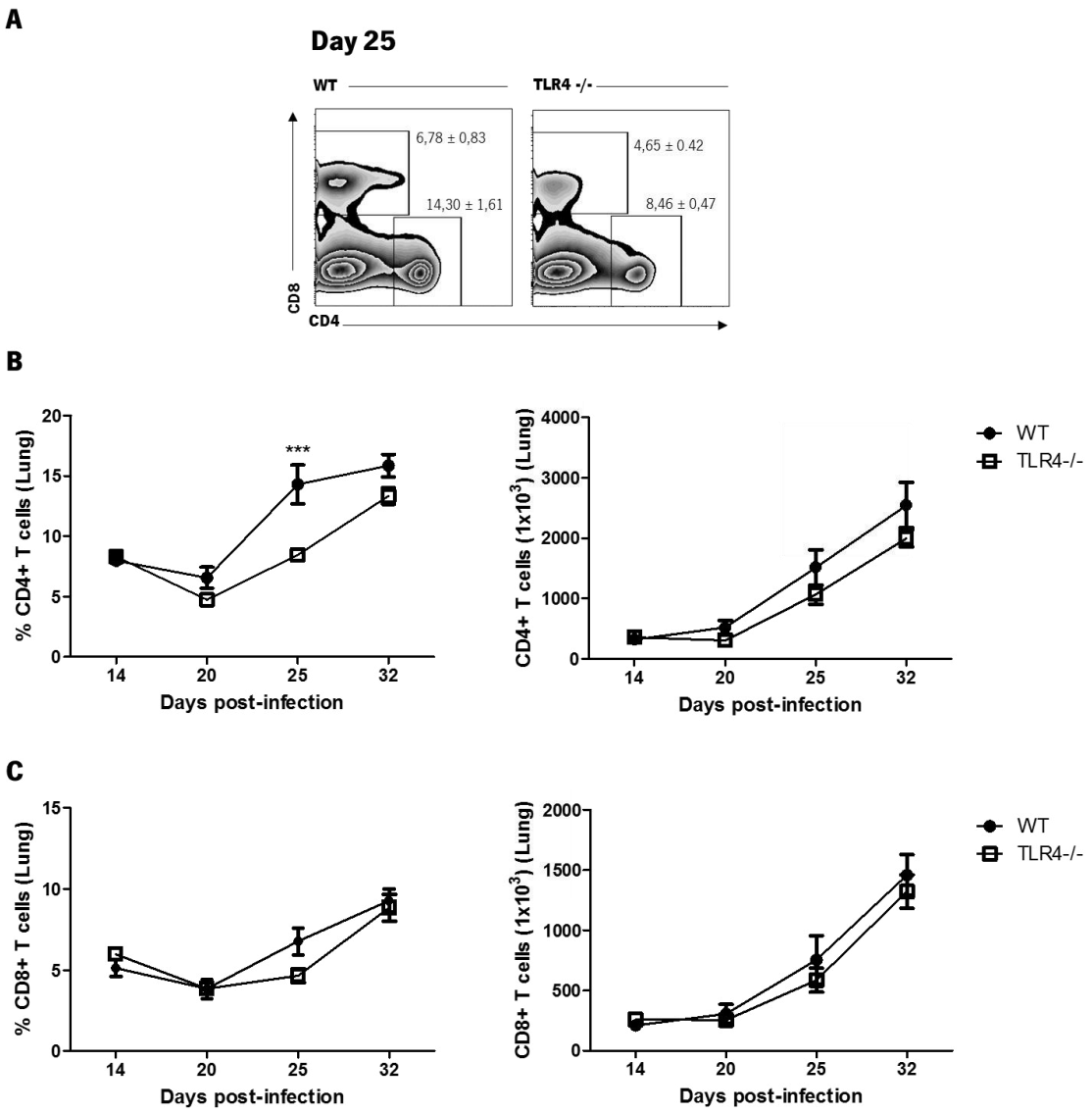


**Figure 1 – TLR4 triggering by 02-171 Mtb strain impacts the frequency of CD4+ T cells at day 25 post-infection.** WT (circles) and TLR4 -/- (squares) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Infected mice were euthanized at the indicated time points after infection. Lung cell suspensions were prepared and the CD4+ and CD8+ T cells were analyzed by flow cytometry. **(A)** Representative FACS profile showing CD4 and CD8 expression by lung cells at day 25 post-infection. The Mean ± SEM of CD4+ and CD8+ cells of each experimental group is indicated. **(B)** Percentage and total number of CD4+ cells in the lung. **(C)** Percentage and total number of CD8+ cells in the lung. The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (***, $p \leq 0.001$)

### 1.2 The increased frequency of CD4+ T cells in the lungs of WT 02-171-infected mice was not due to cell proliferation at the site of infection.

Given that we observed a higher frequency of CD4+ T cells in the lungs of WT mice compared to TLR4 -/- 02-171-infected mice, we questioned whether it was due to higher proliferation of this T cell population in the lungs of WT 02-171-infected mice. In order to assess the proliferation of CD4+ and CD8+ T cells, 24 hours prior the experimental time point, WT and TLR4 -/- 02-171-infected mice were injected with BrdU intraperitoneally, and the percentage of BrdU positive cells – that are actively synthesizing DNA – was determined for each T cell population by flow cytometry (Figure 2). The total number of BrdU positive cells (Figure 2) increased during the course of infection indicating that both CD4+ and CD8+ T cells proliferated in the infected lungs. However, there were no marked differences in the proliferation of CD4+ or CD8+ T cells in WT or TLR4 -/- 02-171-infected mice (Figures 2A and B), suggesting that local T cell proliferation does not account for the difference observed in the frequency of CD4+ T cells in WT versus TLR4 -/- infected lungs (Figure 1B).

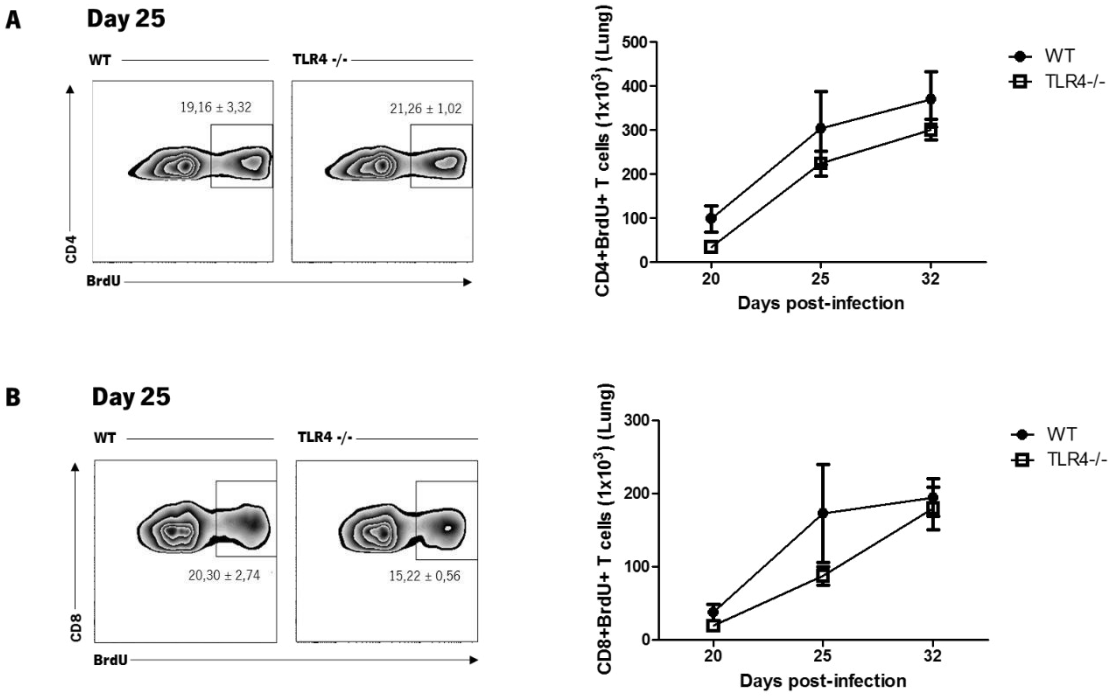


**Figure 2 – Cell proliferation was not responsible for the increased frequency of CD4+ T cells in the lungs of WT 02-171-infected mice.** WT (circles) and TLR4 -/- (squares) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Infected mice were euthanized at indicated time points after infection. Twenty-four hours before the harvesting, mice

were subjected to an IP injection containing 0.8mg of BrdU (100µL/mouse). Lung cell suspensions were prepared and the levels of cell-associated BrdU were measured by flow cytometry. The frequency and total number of BrdU positive cells were analyzed either in CD4+ T cells **(A)** or in CD8+ T cells **(B)**. Representative FACS profile of BrdU expression among CD4+ or CD8+ T cells at day 25 post-infection. The Mean ± SEM of BrdU positive cells of each experimental group is indicated (left panels). The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment.

### 1.3 Increased expression of *Cxcl10* and *Cxcl11* in 02-171-infected WT mice compared to TLR4 -/- mice

Exposure to mycobacteria antigens elicits effector T cell differentiation in local LN, followed by migration of activated T cells to and within the site of infection [4]. In the LN, T cells become activated and committed to a specific lineage such as Th1 or Th17 cells, increasing in their cell surface the expression of specific receptors as CXC chemokine receptor 3 (CXCR3), C-C chemokine receptor 5 (CCR5) and CCR2 [127]. CXCR3 binds to specific chemokines as CXCL9, CXCL10 and CXCL11 [127]. The expression of these chemokines is associated with the increased recruitment of Th1 cells to the local of infection [127].

Our previous results demonstrated that the increased frequency of CD4+ T cells in the lungs of WT 02-171-infected mice comparative to TLR4 -/- mice was not due to local proliferation. Next, we investigated if the difference observed was a consequence of differential recruitment of T cells to the infected lungs. To address that, we analyzed by RT-PCR the relative mRNA expression levels of CXCR3 specific chemokines namely CXCL9, CXCL10 and CXCL11 (Figure 3). The expression of these Th1 cell recruiting chemokines was increased in the infected lungs as early as 20 days post-infection which coincides with the arrival to and accumulation of T cells in the infected lungs (Figure 1B). Consistent with the higher frequency of CD4+ T cells at day 25 post-infection in WT-infected mice compared to TLR4 -/- mice, the relative mRNA expression levels of *Cxcl10* (Figure 3B) and *Cxcl11* (Figure 3C) at day 20 post-infection were significantly higher in the lungs of WT-infected mice than in TLR4 -/- -infected ones. Although not statistically significant, the levels of *Cxcl9* were also higher in WT than in TLR4 -/- infected lungs (Figure 3A). Therefore, these findings suggest that the increased frequency of CD4+ T cells in WT mice could be due to the differential expression of chemokines in the lungs of WT and TLR4 -/- -infected mice, which would contribute to a differential recruitment of these cells.

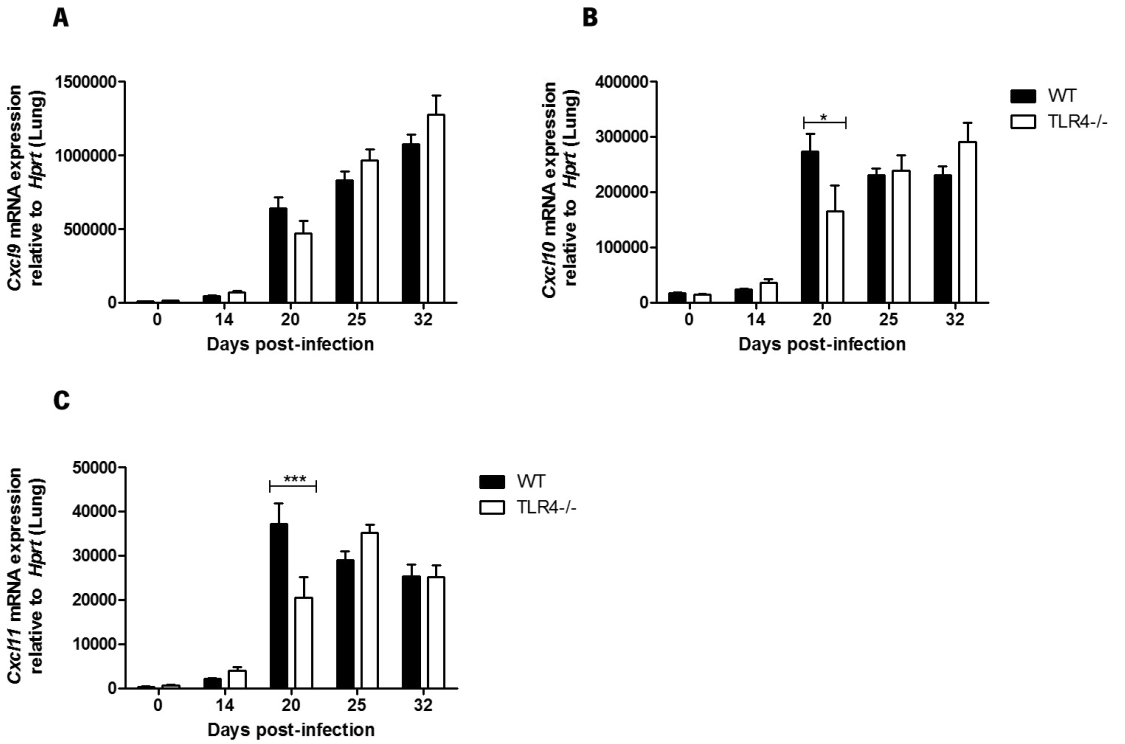


**Figure 3 – Chemokines responsible for attracting T cells were differentially expressed in the lungs of WT and TLR4 -/- mice during 02-171 Mtb infection.** WT (black bars) and TLR4 -/- (white bars) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Mice were euthanized at indicated time points after infection. Total RNA was extracted from lung homogenates and the relative mRNA expression of *Cxcl9* **(A)**, *Cxcl10* **(B)** and *Cxcl11* **(C)** was analyzed by RT-PCR and normalized to the expression of *Hprt*. Data represented for day 0 correspond to uninfected animals. Data represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05; ***, p≤0.001)

## 1.4 TLR4 triggering by 02-171 Mtb strain does not impact the phenotype of CD4+ T cells generated during infection

In addition to the frequency of CD4+ T cells present in the infected lungs, their specific phenotype is also determinant for the protection offered by these cells. For this reason, we next investigated whether TLR4 triggering by a TLR4-activating Mtb strain influenced the phenotype of CD4+ T cells. For that, lung cell suspensions were stained for the surface markers CD69, KLRG-1 and PD-1 and their expression levels analyzed by flow cytometry. CD69 expression has been used as a marker for early T cell activation [10]. During Mtb infection, KLRG1 expressing T cells have a higher ability to produce cytokines, but low proliferative capacity identifying terminally differentiated cytokine-

producing effector T cells. On another hand, PD-1 positive T cells are activated effector cells, less differentiated, with high proliferative and low cytokine production ability [105, 128].

We observed that the number of CD4+ T cells expressing these activation markers at the infection site started to increase around 20 days post-infection (Figure 4). While the number of CD4+ T cells expressing CD69 and PD-1 still increased at least until day 32 post-infection (Figures 4A and C), CD4+ T cells expressing KLRG1, increased from day 20 of infection, achieved the peak at day 25 post-infection and their expression stabilized until day 32 post-infection (Figure 4B). Furthermore, we found similar expression of CD69, KLGR-1 and PD-1 in CD4+ gated T cells of WT or TLR4 -/- 02-171-infected mice (Figures 4A, B and C), supporting no role for TLR4 activation on the phenotypic activation of CD4+ T cells, at least for the markers tested.

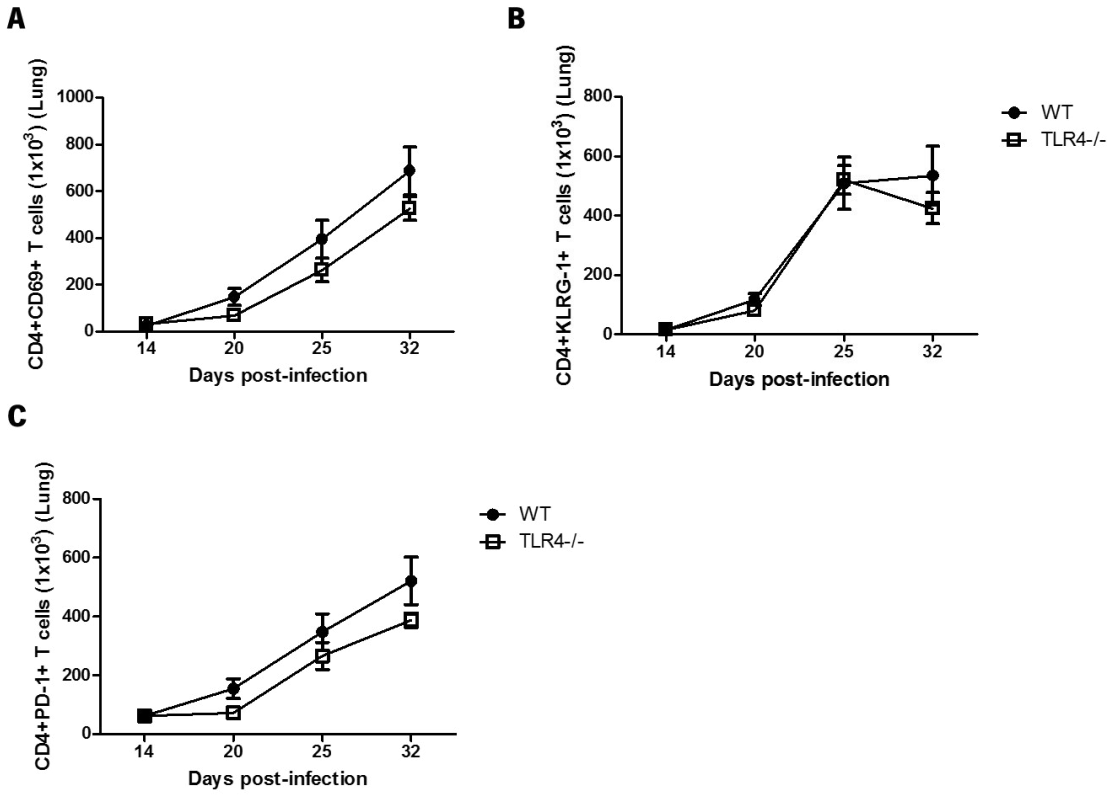


**Figure 4 – Absence of TLR4 signaling during 02-171 Mtb infection does not impact the phenotype of CD4+ T cells.** WT (circles) and TLR4 -/- (squares) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Infected mice were euthanized at indicated time points after infection. Lung cell suspensions were prepared and the phenotype of CD4 positive cells was assessed by flow cytometry. Total numbers of CD4+ T cells positive for CD69 **(A)**, KLRG1 **(B)** or PD-1 **(C)** are shown. The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment.

### 1.5 TLR4 activation does not impact IFN-γ production by CD4+ T cells in the lung of 02-171-infected mice

To obtain further insight into the impact of TLR4 activation during Mtb infection on the phenotype of lung CD4+ T cells, we analyzed the ability of CD4+ T cells from WT or TLR4 -/- mice infected with Mtb strain 02-171 to produce IFN-γ and IFN-γ plus TNF by flow cytometry. We observed no marked differences in percentage and also in total number of IFN-γ+- (Figure 5A) and IFN-γ+TNF+-producing CD4+ T cells from infected lungs (Figure 5B). Furthermore, both T cell populations peaked at day 25 post-infection and then their expression decreased (Figures 5A and B). An exception to this observation was the dynamics of the number of IFN-γ-producing CD4+ T cells that upon 25 days after Mtb infection the expression was maintained relatively constant (Figure 5A).

We also determined by RT-PCR the relative mRNA expression levels of IFN-γ and TNF cytokines in the total lung of infected mice. We observed that the expression of *Ifng* and *Tnf* increased after 20 days of infection (Figures 5C and D). There were no significant differences in the amounts of *Ifng* or *Tnf* expression in the presence or absence of TLR4 signaling until day 25 post-infection (Figures 5C and D). However, at day 32 post-infection TLR4 -/- mice exhibited higher expression of *Ifng* compared to WT-infected ones (Figure 5C). This could be indicative that in TLR4 -/- mice other cell types must contribute for the overall production of IFN-γ.

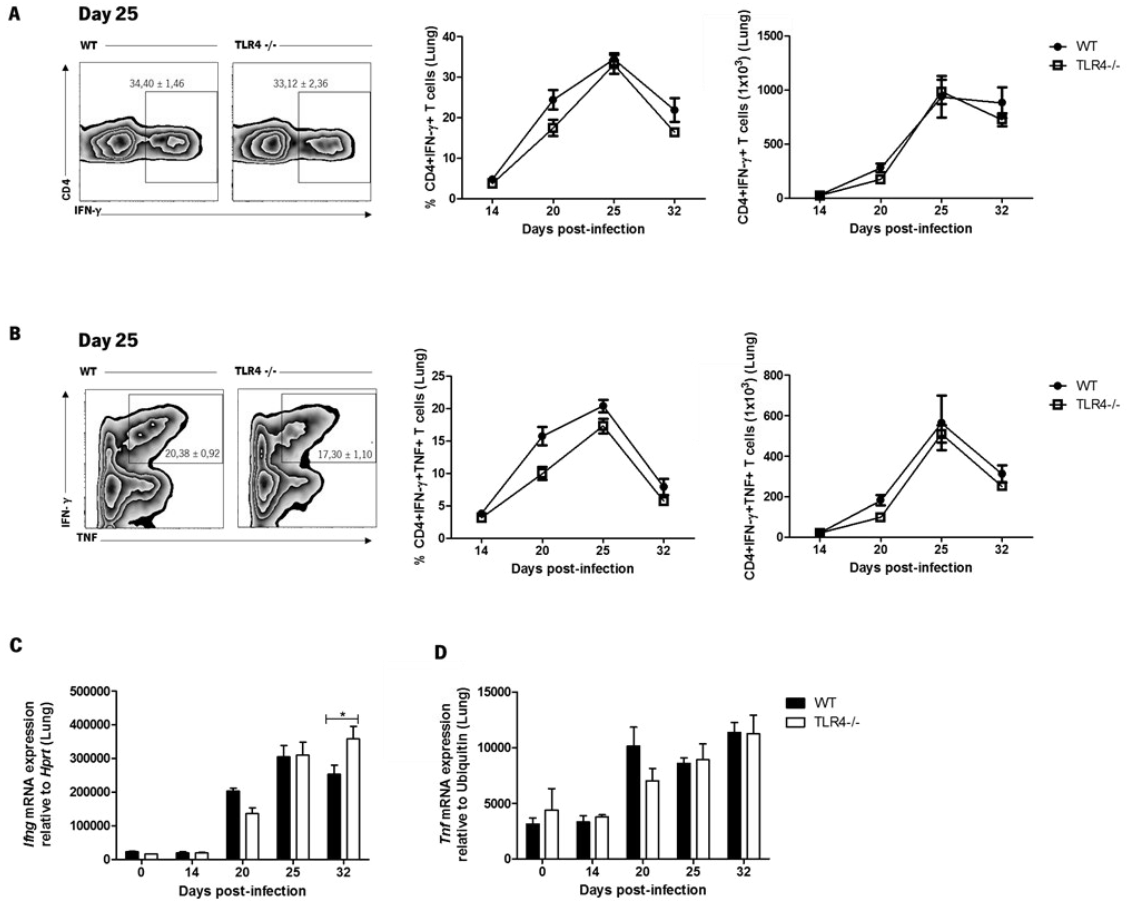


**Figure 5 – TLR4 absence does not impact IFN-γ response in lung CD4+ T cells during 02-171 infection.**

WT (circles/black bars) and TLR4 -/- (squares/white bars) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Mice were euthanized at indicated time points after infection. **(A and B)** Lung cell suspensions were prepared and cytokine production analyzed by flow cytometry. **(A)** Representative FACS profile of IFN-γ at day 25 post-infection. The Mean ± SEM of IFN-γ positive cells of each experimental group is indicated (left panel). Percentage and total number of IFN-γ-producing CD4+ T cells (middle and right panels). **(B)** Representative FACS profile of IFN-γ+TNF+ at day 25 post-infection. The Mean ± SEM of IFN-γ+TNF+ cells of each experimental group is indicated (left panel). Percentage and total number of IFN-γ+TNF+ cells on gated CD4+ T cells (middle and right panels). **(C and D)** Total RNA was extracted from lung homogenates and the relative mRNA expression of *Ifng* **(C)** and *Tnf* **(D)** were analyzed by RT-PCR and normalized to the expression of *Hprt* or ubiquitin as indicated. Data represented for day 0 correspond to uninfected animals. Data represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05)

### 1.6 TLR4 activation impacts IL-17 production by CD4+ T cells in the lung of 02-171-infected mice

In addition to IFN-γ-producing T cells upon Mtb infection, IL-17-producing T cells are also induced [80]. For that reason, we next analyzed by flow cytometry the production of IL-17 or of IL-17 plus TNF by CD4+ T cells from WT or TLR4 -/- mice infected with Mtb strain 02-171. We found that although the production of IFN-γ by CD4+ T cells of 02-171 infected mice was not significantly altered (Figure 5A), the frequency of lung IL-17-producing CD4+ T cells was significantly increased in TLR4 -/- infected mice compared with WT-infected mice at days 20 and 25 post-infection (Figure 6A). However, these differences were not reflected to the total number of IL-17-producing CD4+ T cells (Figure 6A). Moreover, at day 25 post-infection we observed a transient increase in the frequency and in the cell number of IL-17+TNF+ -producing CD4+ T cells in TLR4 -/- infected mice compared with WT mice (Figure 6B). We also determined by RT-PCR the relative mRNA expression levels for *Il17* in the lungs of infected mice. We did not observe any significant difference in the total *Il17* mRNA expression (Figure 6C) between WT and TLR4 -/- infected mice. This is in contrast to what was observed by flow cytometry, which may be explained by the contribution of other cell types for the production of IL-17. Since that IL-23 is fundamental for the maintenance of IL-17-producing T cells [80, 91, 93] we also analyzed by RT-PCR the two subunits of this heterodimeric cytokine namely *Il23a* and *Il12b* [91]. In accordance with *Il17* mRNA expression, we did not observe differences in the relative mRNA expression of *Il23a* (p19) and *Il12b* (p40) (Figure 6D and E), suggesting that TLR4 activation does not appear to influence the expression of IL-23.

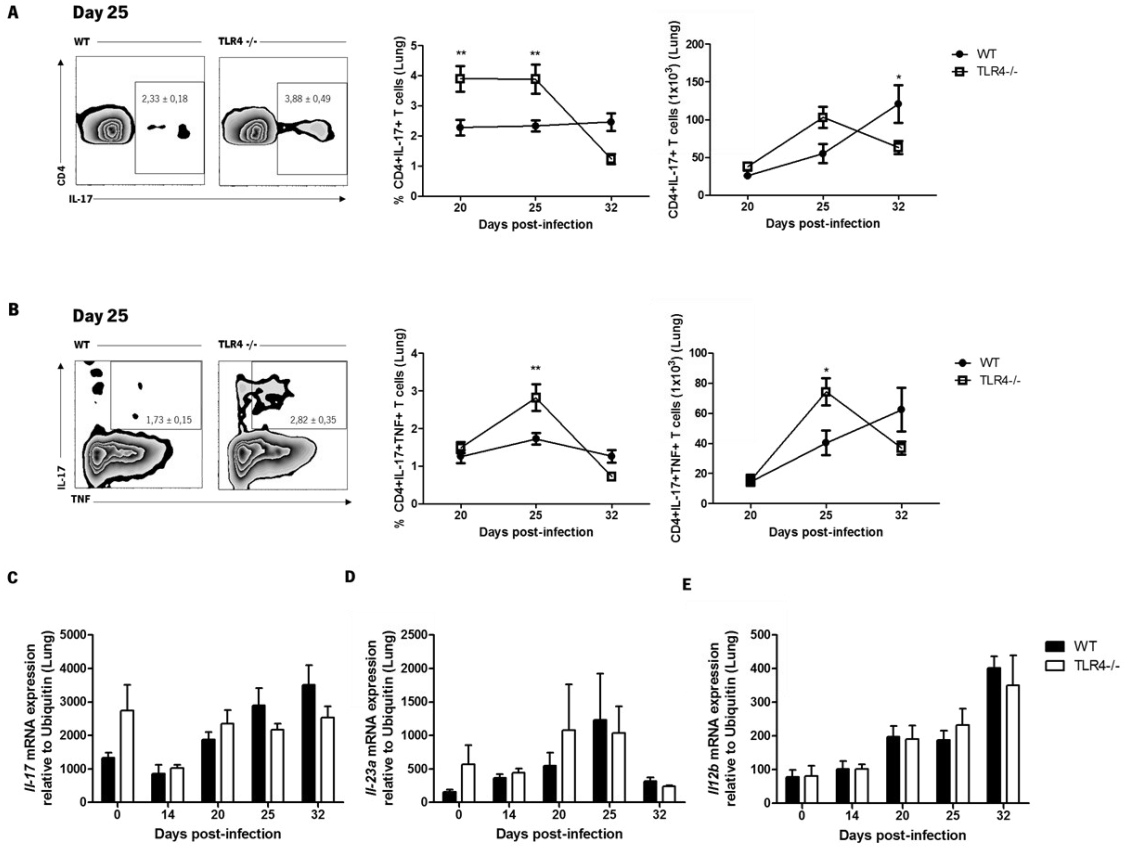


**Figure 6 – TLR4 deficiency influences the frequency of lung IL-17 CD4+ T cell response.** WT (circles/black bars) and TLR4 -/- (squares/white bars) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Mice were euthanized at indicated time points after infection. **(A and B)** Lung cell suspensions were prepared and cytokine production analyzed by flow cytometry. **(A)** Representative FACS profile of IL-17 at day 25 post-infection. The Mean ± SEM of IL-17 positive cells of each experimental group is indicated (left panel). Percentage and total number of IL-17-producing CD4+ T cells (middle and right panels). **(B)** Representative FACS profile of IL-17+TNF+ at day 25 post-infection. The Mean ± SEM of IL-17+TNF+ positive cells of each experimental group is indicated (left panel). Percentage and total number of IL-17+TNF+ CD4+ T cells (middle and right panels). **(C-E)** Total RNA was extracted from lung homogenates and the relative mRNA expression of *Il17* **(C)**, *Il23a* **(D)** and *Il12b* **(E)** were analyzed by RT-PCR and normalized to the expression of ubiquitin. Data represented for day 0 correspond to uninfected animals. Data represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05; **, p≤0.01)

## 1.7 Activation of TLR4 by 02-171 Mtb strain during primary infection does not impact lung neutrophil accumulation neither lung inflammation

It has been reported that IL-17 production induces early neutrophil recruitment that might lead to tissue damage in cases of exacerbated IL-17 response [80]. Since we observed an increased

frequency of IL-17-producing CD4+ T cells in TLR4 -/- mice comparatively to WT infected mice, we next investigated the consequences of this increment for the lung inflammatory response. Thus, firstly we assessed the neutrophil accumulation in the lungs by flow cytometry. The frequency and total number of neutrophils (CD11b+Ly6G+ cells) were statistically similar between WT and TLR4 -/- 02-171-infected mice (Figure 7A), although apparently higher in TLR4 -/- mice at day 25 post-infection. It is thus possible that the increase in IL-17 impacts lung neutrophil recruitment, albeit at the time points tested this did not reach statistical significance.

We also evaluated the role of TLR4 signaling on lung inflammation. For that, lung sections of the upper right lobe of infected mice were stained with H&E and analyzed. At day 20 post-infection we observed the appearance of small granulomata. From this time point onwards, the inflammatory infiltrates become more intense, until day 32 when we ended the experiment (Figure 7B). In accordance with the neutrophils counts, we did not observe significant differences in lung inflammation between WT and TLR4 -/- infected mice for the analyzed time points (Figure 7B). However, a quantitative analysis needs to be performed to achieve definitive conclusions.

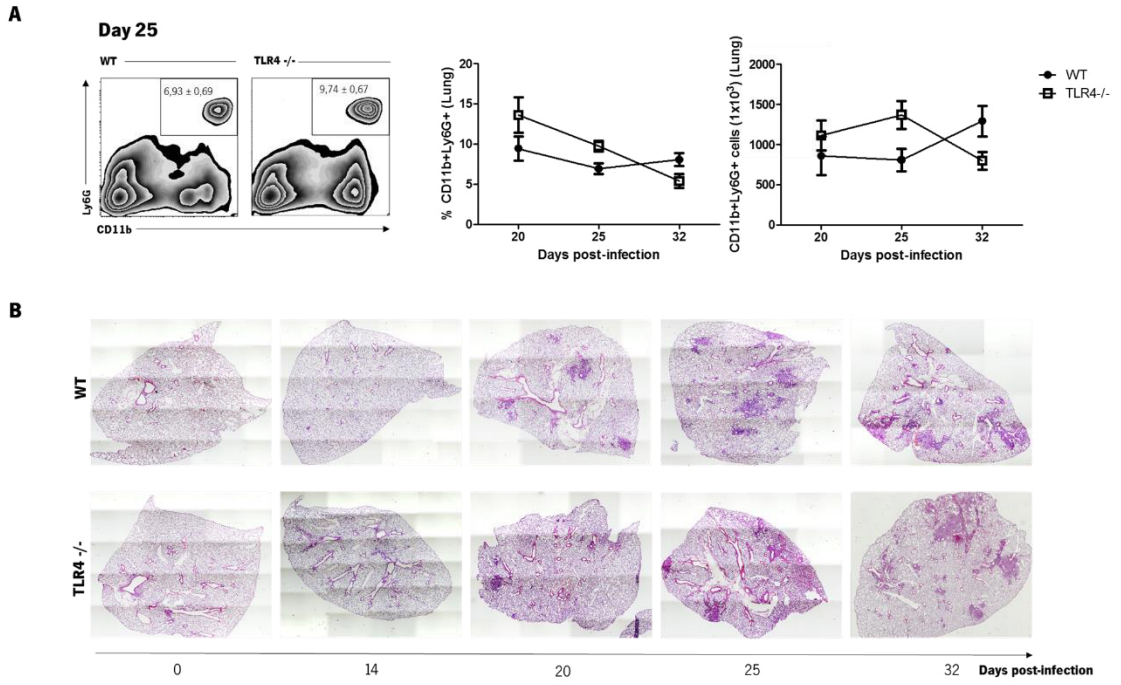


**Figure 7 – TLR4 triggering by 02-171 Mtb strain does not impact lung neutrophil accumulation neither lung inflammation.** WT (circles) and TLR4 -/- (squares) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Mice were euthanized at indicated time points after infection. **(A)** Lung cell suspensions were prepared and the neutrophils were analyzed by the CD11b and Ly6G expression by flow cytometry. Representative FACS profile of neutrophils (CD11b+Ly6G+) at day 25 post-infection. The Mean ± SEM of CD11b+Ly6G+ cells of each experimental group is indicated (left panel).

Percentage and total number of CD11b+Ly6G+ cells in the lung (middle and right panels). The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. **(B)** To assess lung inflammation the upper right lobes of the lung were excised from WT (top panel) or TLR4 -/- (bottom panel) mice and fixed lung sections were stained with H&E. Representative images from each experimental group were obtained. Images represented for day 0 correspond to uninfected animals. Data are from one experiment.

### 1.8 TLR4 activation by 02-171 Mtb strain does not impact *Nos2* expression

Since we have previously described that upon intranasal infection with 02-171 Mtb strain the TLR4 deficiency compromises NOS2 expression at gene and protein level [34], we next questioned if we also obtained this difference using the aerosol model and a low dose of infection. For that, we measured the induction of NOS2 in lung homogenates at gene expression level by RT-PCR and in lung tissues at protein level by immunofluorescence.

As previously reported, we observed an increased expression of NOS2 during the progression of infection (Figure 8). Furthermore, we only detected considerable levels of NOS2 both at gene and protein level from day 20 post-infection (Figures 8A and B) which coincides with the accumulation of IFN-γ-producing cells in the lung (Figures 5A and C). In contrast with what we found before using intranasal model of infection [34], we observed statistically similar levels of *Nos2* expression (Figure 8A) and NOS2 protein (Figure 8B) in both groups of Mtb-infected mice, although apparently higher in WT mice at day 20 post-infection (Figures 8A and B). However, a quantitative analysis needs to be performed to achieve definitive conclusions. Given that IFN-γ and TNF signaling synergize to the induction of NOS2 during Mtb infection [5, 56], our data is in line with our previous findings that IFN-γ and TNF were also not affected in the absence of TLR4 (Figure 5A, C and D).

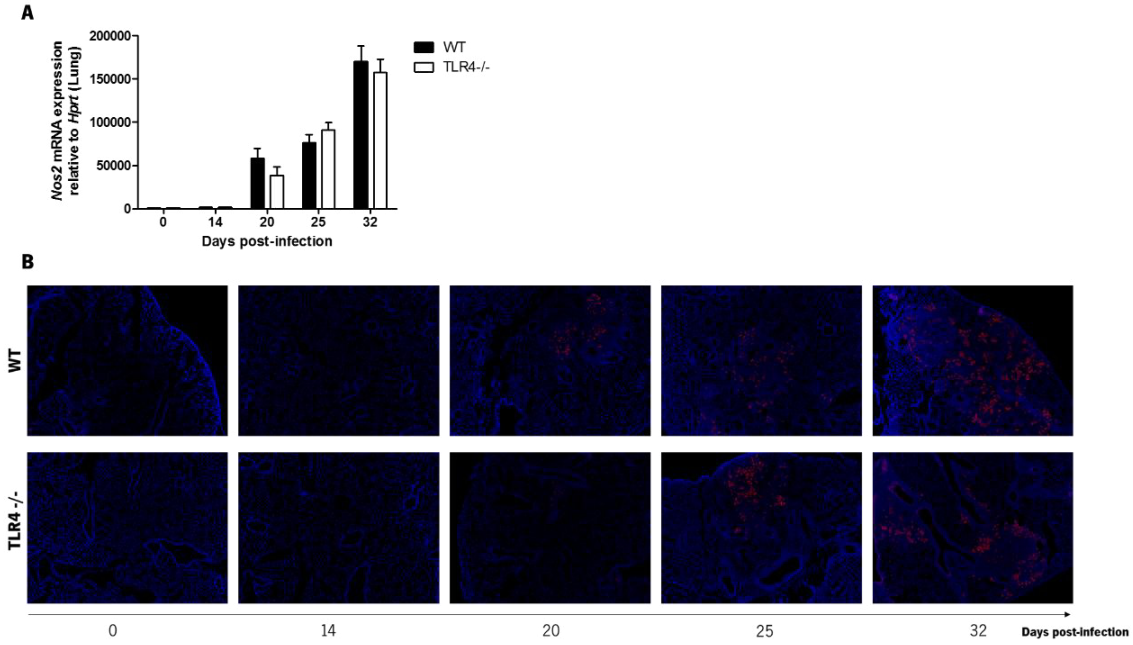


**Figure 8 – TLR4 triggering by 02-171 Mtb strain does not impact Nos2 mRNA neither NOS2 protein expression.** WT (circles) and TLR4 -/- (squares) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.155 ± 0.015 (Mean ± SEM for 4 animals)). Mice were euthanized at indicated time points after infection. **(A)** Total RNA was extracted from lung homogenates and the relative mRNA expression of *Nos2* was analyzed by RT-PCR and normalized to the expression of *Hprt*. Data represented for day 0 correspond to uninfected animals. Data represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. **(B)** The upper right lobes of the lung were excised from WT (top panel) or TLR4 -/- (bottom panel) mice and lung sections were used to detect NOS2 protein by immunofluorescence. Images were obtained with 4x magnification and were representative from each experimental group. NOS2 protein (red) and DAPI (blue). Images represented for day 0 correspond to uninfected animals. Data are from one experiment.

## 1.9 TLR4 deficiency does not compromise protection against an aerosol infection with a low dose of TLR4-activating 02-171 Mtb strain

We have reported that the absence of TLR4 led to an increased bacterial burden in the lungs of 02-171 intranasally infected mice. The same did not occur for H37Rv intranasally infected mice, which suggested a protective role for TLR4 activation upon infection with a TLR4-activating Mtb strain [34].

Taking into account that upon aerosol infection with a low dose of 02-171 we did not observe differences in the expression of protective cytokines (e.g. IFN-γ and TNF) neither in *Nos2* expression in WT and TLR4 -/- mice, we determined the bacterial burdens in the lungs and in the LN of WT

and TLR4 -/- mice after aerosol infection with 02-171 Mtb strain. This would help to elucidate if the loss of differences among the intranasal and aerosol infections could be reflected in the progression of infection in those mice.

As published before, the bacterial burden in the lung of Mtb-infected mice increases exponential until arrival of T cells to the local of infection [4], which happens around 14 days post-infection. Then, the bacterial growth slows with the accumulation of T cells [4] which in our experiment occurred between day 14 and 20 post-infection (Figure 9A), and finally reached a stable plateau around day 30-32 post-infection (Figure 9A). In the LN the bacterial burden peaked at day 20 post-infection, which might suggest the dissemination of the mycobacteria from the lung to the LN and the initiation of the T cell priming (Figure 9B). We did not observe any difference in the bacterial burden in the lungs and LN between TLR4 -/- and WT aerosol 02-171-infected mice (Figures 9A and B). Although these data are in apparent contrast with our previous work [34], these conflicting results can possibly be explained by the different route of infection (intranasal vs aerosol) and/or by the initial inoculum dose (low vs high infection dose).

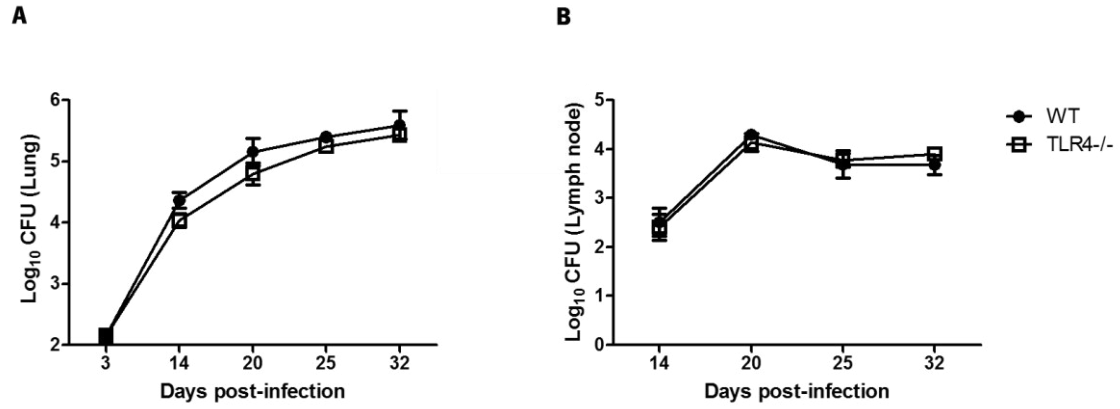


**Figure 9 – TLR4 deficiency does not impact bacterial growth after aerosol infection with 02-171 Mtb strain.** WT (circles) and TLR4 -/- (squares) mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection. Infected mice were euthanized at indicated time points after infection and serial dilutions of lung **(A)** and LN **(B)** homogenates were plated and the bacterial burden were determined after 21 days of incubation. The initial infection dose in the lungs was $Log_{10}$(CFUs) 2.155±0.015 (Mean ± SEM for 4 animals). The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment.

## 1.10 Initial infectious dose impacts susceptibility of NOS2 -/- mice to the 02-171 Mtb infection

We hypothesized that the higher susceptibility observed in TLR4 -/- mice compared with WT mice intranasally infected with 02-171 can be due to the inoculum dose, since the initial bacterial burden in the lungs of mice infected via the intranasal route was higher 380.19 ± 2.69 CFUs per mice (Mean ± SEM for 6 animals) [34] than in the experiment reported in this work 143.25 ± 5.14 CFUs per mice (Mean ± SEM for 4 animals) (Figure 9A).

We have data in our laboratory from WT and NOS2 -/- mice which are in agreement with our hypothesis. WT and NOS2 -/- mice were infected via the aerosol route with different doses of 02-171 Mtb strain by modulating the concentration of bacteria placed in the nebulizer. At day 3 post-infection mice were sacrificed to determine the initial dose of bacteria delivered to the lung. The group of mice with the lowest dose was exposed to a $Log_{10}$(CFU) 1.814±0.049 (Mean ± SEM for 4 animals) corresponding to an average of 66.50 ± 7.96 CFUs per mice (Figure 10A) and the group of mice with the standard dose was exposed to a $Log_{10}$(CFUs) 2.008±0.048 (Mean ± SEM for 5 animals) that correspond to an average of 104.00 ± 11.10 CFUs per mice (Figure 10B). NOS2 -/- mice infected with the higher inoculum were less protected than WT mice as soon as 18 days post-infection (Figure 10B). Whereas, with the lower inoculum dose NOS2 -/- mice only became more susceptible than WT 02-171-infected mice at day 31 post-infection (Figure 10A). These observations support our hypothesis that the inoculum size can influence the outcome of *in vivo* infection of mice with the TLR4-activating 02-171 Mtb strain. Of note, another major difference is the route of infection, which we have still to experimentally address.

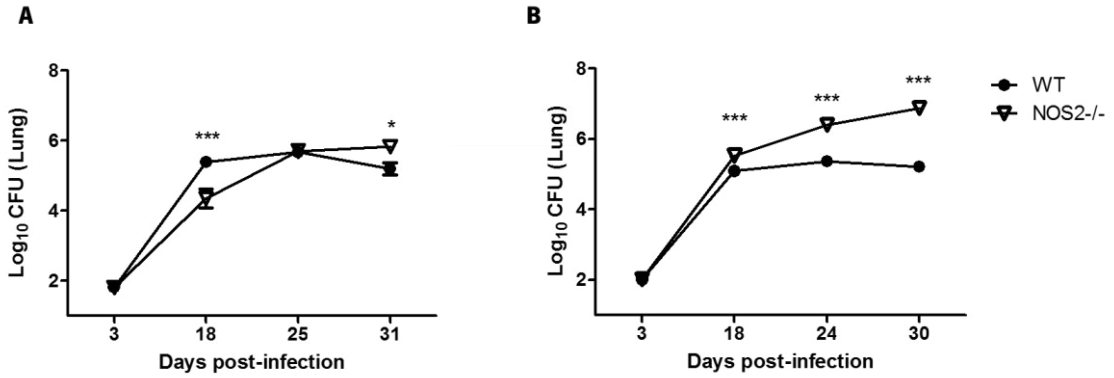


**Figure 10 – Higher infectious dose results in higher susceptibility of NOS2 -/- mice to the 02-171 Mtb infection.** WT (circles) and NOS2 -/- (inverted triangles) mice were infected with Mtb strain 02-171 via the aerosol

route with a low dose of infection. Infected mice were euthanized at indicated time points after infection and serial dilutions of lung homogenates were plated and the bacterial burden were determined after 21 days of incubation. The initial infection dose in the lungs in **(A)** and **(B)** was $Log_{10}$ (CFUs) 1.814±0.049 (Mean ± SEM for 4 animals) and $Log_{10}$ (CFUs) 2.008±0.048 (Mean ± SEM for 5 animals), respectively. The initial infection dose was statistical different with $p \leq 0.05$ ($p=0.0352$). The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, $p \leq 0.05$; ***, $p \leq 0.001$)

# RESULTS – CHAPTER II

## EFFICACY OF BCG VACCINATION UPON INFECTION WITH A TLR4-ACTIVATING MTB STRAIN: ROLE OF TLR4 ACTIVATION DURING SECONDARY INFECTION

It is well established that protection conferred by BCG against pulmonary TB is highly variable [101, 102]. Moreover, based on increasing evidences on the genetic heterogeneity and phenotypic differences among Mtb strains [15, 129], it was hypothesized that differences in strain genetics could also be partly responsible for the variation in BCG efficacy. In support of this hypothesis, recent studies have shown that BCG is less effective to protect mice against challenge with certain Beijing strains than with the laboratory strain H37Rv [19, 21].

Considering the role of TLRs in shaping acquired immunity [52, 53], we hypothesized that the differential TLR recognition of Mtb strains, which we defined as a molecular basis for heterogeneous immune responses to Mtb, may also be the basis for the variable efficacy of BCG.

## 2.1 BCG vaccination protects WT mice against an infection by a Beijing TLR4-activating Mtb strain

Given that BCG is mainly recognized by TLR2 [126], we speculated that the type of acquired immunity induced by BCG immunization may be protective against TLR2-activating Mtb strains, as H37Rv, but defective towards a TLR4-activating Mtb strain challenge. To test our hypothesis that the differential TLR recognition of Mtb strains could also account for the variability of BCG protection, we vaccinated WT mice with BCG 90 days prior to 02-171 Mtb strain challenge, a period of time that was previously shown in our laboratory to confer optimal protection against H37Rv infection (A. Cruz *et al.*, submitted for publication).

At specific time points post-infection, mice were sacrificed and the bacterial burden were determined after 21 days of incubation, by plating serial dilutions of lung and liver homogenates.

As shown in Figure 11A, BCG vaccinated mice were protected against aerosol infection with 02-171 Mtb, as shown by the reduction of bacterial burden in the lungs, the primary site of infection. The protection was also observed in the liver that represents a local of dissemination of Mtb infection (Figure 11B). BCG vaccination limited the bacterial growth in the lungs and in the liver as soon as 14 days post-infection (Figure 11A and B), similarly to the protection obtained for Mtb infection in other reported experimental mouse models [103]. This suggests that BCG vaccination accelerates the protective T cell response, causing an early limitation of 02-171 bacterial expansion as it has been reported for H37Rv [103]. Moreover, we found that BCG immunization can maintain long-lasting control of 02-171 Mtb strain infection since the infection remained controlled at day 90 post-infection, being the bacterial burden in the lungs of vaccinated mice significantly lower than that exhibited by unvaccinated mice (Figure 11A). These findings indicate that BCG

vaccination can protect WT mice against an infection caused by the Beijing 02-171 Mtb strain. Our data does not support the previously suggested observation that BCG does not afford protection against infection with Mtb strains from the Beijing lineage [19, 21].

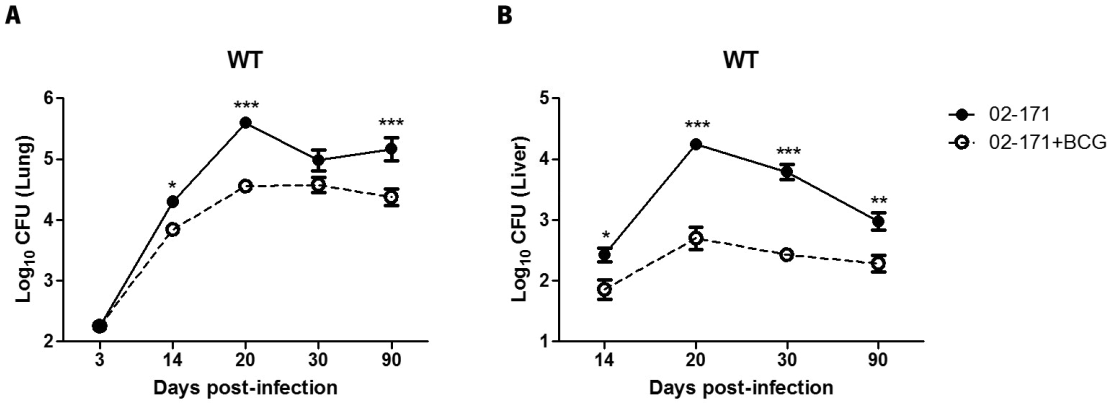


**Figure 11 – BCG immunization induces protective immunity against a Beijing TLR4-activating Mtb strain in WT mice.** BCG vaccinated (02-171+BCG) (open circles, dashed line) or unvaccinated (02-171) (closed circles, solid line) WT mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection. At the indicated time points after infection mice were sacrificed and the bacterial burden were determined after 21 days of incubation by plating serial dilutions of lung **(A)** and liver **(B)** homogenates. The initial infection dose into the lungs was $Log_{10}$(CFUs) 2.250±0.037 (Mean ± SEM for 5 animals). The data points represent the Mean ± SEM of five mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05; **, p≤0.01; ***, p≤0.001)

## 2.2 BCG-induced protection associates with an anticipation of CD4+ T cell response in the lungs of WT mice

To gain insight into the mechanisms underlying BCG protection against 02-171 infection and given that CD4+ T cell responses have been shown of utmost importance in the control of Mtb infection [5, 56], we next compared by flow cytometry the CD4+ T cell response in the lungs of vaccinated and unvaccinated WT mice after aerosol challenge with a low dose of 02-171 Mtb strain. We observed an anticipation in the accumulation of both frequency and number of CD4+ T cells, on day 14 post-infection in the lungs of vaccinated WT mice compared with unvaccinated WT mice (Figure 12A and B). This increment was lost at day 20 after infection, with CD4+ T cell numbers becoming similar in both groups of infected mice after 20 days of infection (Figure 12B). In unvaccinated WT mice the number of CD4+ T cells in the lungs remained relatively constant until day 20 post-infection from which began to rise, peaking at day 30 post-infection and decreasing

thereafter (Figure 12B). During primary infection, T cell responses arrive to the site of infection around day 20 post-infection (Figure 12B) when Mtb infection starts to be controlled (Figure 11A). These data suggest that the higher numbers of CD4+ T cells at day 14 post-infection in vaccinated compared to unvaccinated WT mice (Figure 12B) associates with the better control of 02-171 Mtb growth in BCG immunized mice (Figure 11A), as it has been reported for H37Rv infection [103].

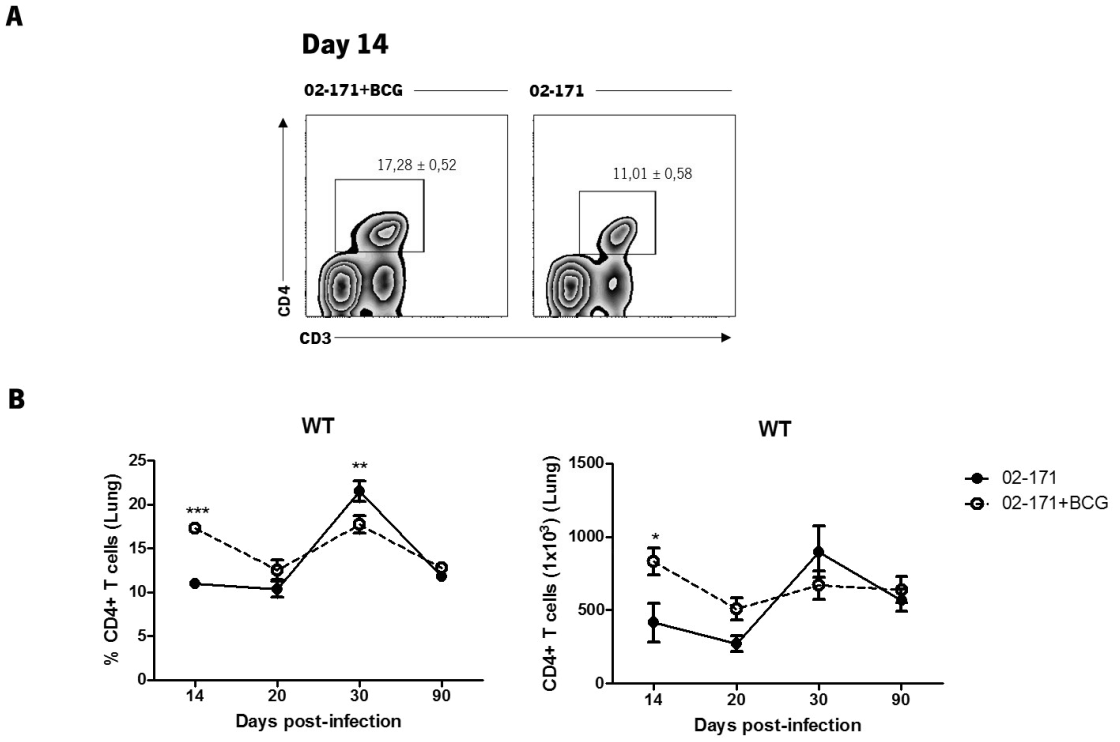


**Figure 12 – Increased frequency and number of lung CD4+ T cells at day 14 of infection in BCG vaccinated WT mice.** BCG vaccinated (02-171+BCG) (open circles, dashed line) or unvaccinated (02-171) (closed circles, solid line) WT mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.250 ± 0.037 (Mean ± SEM for 5 animals). Infected mice were euthanized at the indicated time points after infection. Lung cell suspensions were prepared and the CD4+ T cells were analyzed by flow cytometry. **(A)** Representative FACS profile showing CD4 expression by lung cells at day 14 post-infection. The Mean ± SEM of CD4 positive cells of each experimental group is indicated. **(B)** Percentage and total number of CD4 positive cells in the lung. The data points represent the Mean ± SEM of five mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05; **, p≤0.01; ***, p≤0.001)

## 2.3 BCG-mediated control of 02-171 Mtb infection associates with an early accumulation of IFN-γ+TNF+IL-2+ multifunctional CD4+ T cell response in the lungs of WT mice

In order to characterize the phenotype of CD4+ T cell responses developed in BCG vaccinated 02-171 infected mice, we compared by flow cytometry the ability of CD4+ T cells to produce IFN-γ in the lungs of vaccinated and unvaccinated WT mice after aerosol challenge with 02-171 Mtb strain. BCG vaccinated WT mice exhibited a higher frequency of lung IFN-γ-producing CD4+ T cells at day 14 after infection compared with WT 02-171-infected mice (Figure 13A and B). Although without statistical significance, we found the same tendency at this time point for the number of IFN-γ-producing CD4+ T cells (Figure 13B). In WT vaccinated mice, both frequency and number of IFN-γ CD4+ T cells, was already increased at day 14 of infection, raised from day 20 after infection, peaked at day 30 and then decreased becoming similar to WT unvaccinated mice (Figure 13B). In unvaccinated WT mice, the accumulation of IFN-γ-producing CD4+ T cells in the lungs happened later on, with IFN-γ-producing CD4+ T cells increasing from day 20 after infection, peaking at day 30 post-infection and at day 90 the IFN-γ-producing CD4+ T cells are already decreased (Figure 13B). Therefore, the early IFN-γ response induced by BCG vaccination associates with the early Mtb growth arrest in WT BCG vaccinated mice (Figures 11A and 13B). Furthermore, at day 30 of Mtb infection, unvaccinated WT mice exhibited a higher frequency and number of IFN-γ-producing CD4+ T cells than vaccinated WT mice, which again correlates with the control of Mtb infection in those mice (Figures 11A and 13B). However, at this time point the difference in IFN-γ-producing CD4+ T cells in unvaccinated WT mice compared to vaccinated mice does not seem associate with an increased protection in the infected lungs of unvaccinated WT mice compared with vaccinated WT mice (Figures 11A and 13B).

As previously mentioned, IFN-γ T cell responses play a critical protective role during Mtb infection [4, 5, 56, 86]. However the expression of this cytokine is not, by itself, sufficient to confer protection [130, 131]. It has been reported that the accumulation of multifunctional CD4+ T cells generated during vaccination that express IFN-γ in combination with TNF and IL-2 associate with increased protection against Mtb infection [130, 131]. Owing to the relevance of IFN-γ-producing multifunctional CD4+ T cells in vaccine-induced protection, we analyzed by flow cytometry the co-expression of IFN-γ, TNF and IL-2 by lung CD4+ T cells from 02-171 infected and BCG vaccinated or unvaccinated mice. The frequency of IFN-γ+TNF+IL-2+ CD4+ T cells was significantly higher in unvaccinated WT mice at day 14 post-infection, compared with vaccinated ones (Figure 13C and

D). After this time point the frequency of those CD4+ T cells dropped until day 20 after infection, becoming similar in both vaccinated and unvaccinated WT mice (Figure 13D). WT BCG vaccinated mice showed an increased number of lung CD4+ T cells co-producing IFN-γ, TNF and IL-2 at day 14 post-infection compared with unvaccinated WT mice (Figure 13D). After this time point the number of this multifunctional CD4+ T cell population dropped until day 20 after infection, becoming similar to WT unvaccinated mice (Figure 13D). IFN-γ+TNF+IL-2+ T cells peaked at day 30 post-infection, decreasing thereafter, in both groups of WT mice (Figure 13D). In accordance with the IFN-γ CD4+ T cell response, we also observed an early induction and accumulation of IFN-γ+TNF+IL-2+ multifunctional CD4+ T cells in vaccinated mice that might account for the better control of 02-171 Mtb infection in these mice.

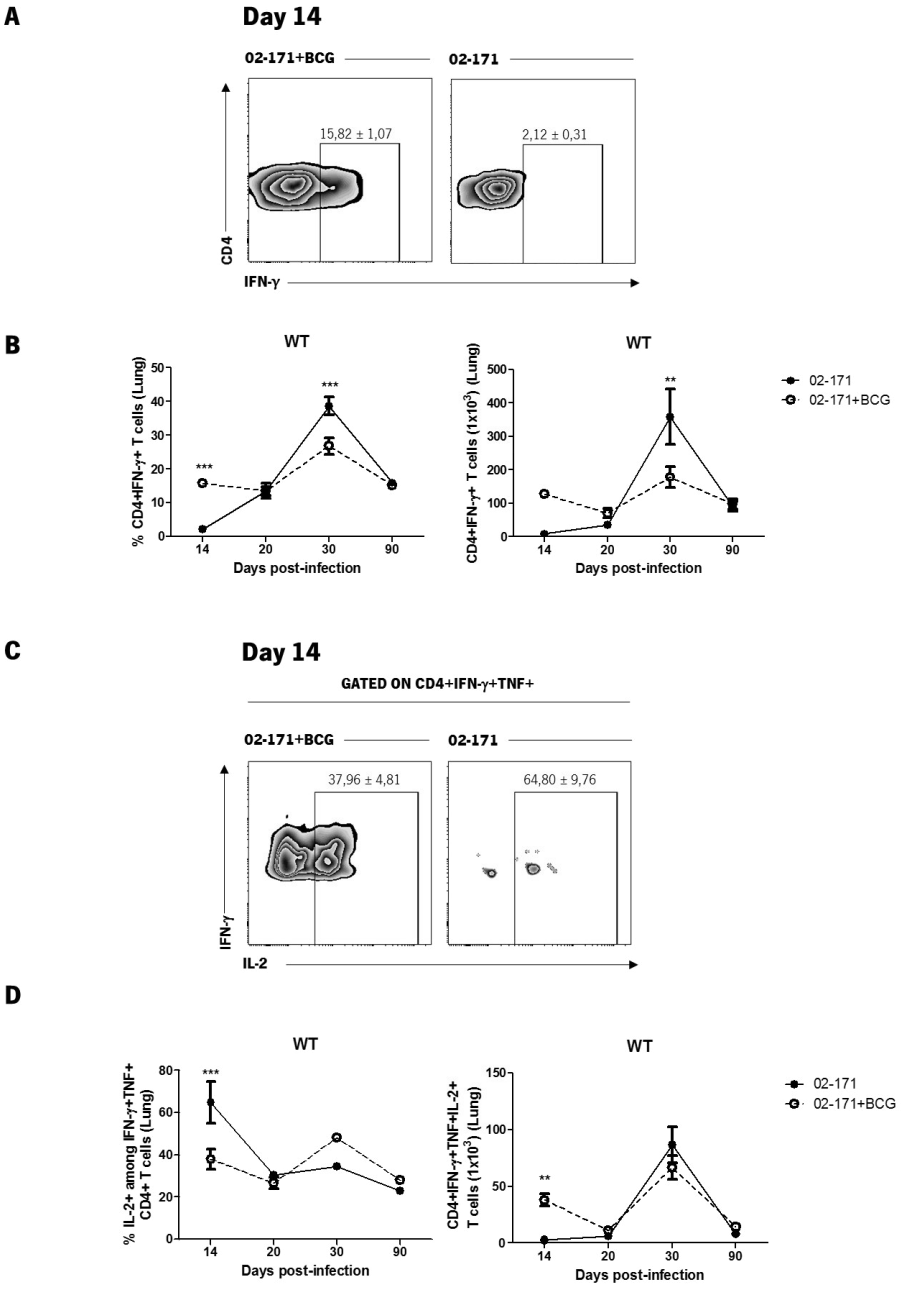


**Figure 13 – BCG vaccination leads to an early accumulation of lung IFN-γ+TNF+IL-2+ multifunctional CD4+ T cells after Mtb infection.** BCG vaccinated (02-171+BCG) (open circles, dashed line) or unvaccinated (02-171) (closed circles, solid line) WT mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.250 ± 0.037 (Mean ± SEM for 5 animals). Infected mice were euthanized at the indicated time points after infection. Lung cell suspensions were prepared and cytokine production were analyzed by flow cytometry. **(A)** Representative FACS profile showing IFN-γ expression by lung CD4+ T cells at day 14 post-infection. The Mean ± SEM of IFN-γ positive cells of each experimental group is indicated. **(B)** Percentage and total number of IFN-γ positive cells on gated CD4+ T cells. **(C)** Representative FACS profile showing the expression of IL-2+ among IFN-γ+TNF+ by lung CD4+ T cells at day 14 post-infection. The Mean ± SEM of IFN-γ+TNF+IL-2+ cells of each experimental group is indicated. **(D)** Percentage and total number of IFN-γ+TNF+IL-2+ on gated CD4+ T cells. The

data points represent the Mean ± SEM of five mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (**, p≤0.01; ***, p≤0.001)

## 2.4 BCG-mediated control of 02-171 Mtb infection associates with an increased induction of IL-17+TNF+IL-2+ multifunctional CD4+ T cell responses in the lungs of WT mice

It has been previously described that Th17 cells are important for BCG-induced protection by recruiting protective IFN-γ-producing CD4+ T cells to the lungs [103]. Based on that, we next investigated, by flow cytometry, the kinetics of IL-17-producing CD4+ T cells in the lungs of vaccinated and unvaccinated WT mice after 02-171 aerosol challenge.

Upon aerosol infection with 02-171 Mtb strain, we observed an increased frequency and number of IL-17+ CD4+ T cells in the lungs of BCG vaccinated, WT mice but not in unvaccinated, WT mice (Figure 14A). The increased numbers of IL-17-producing CD4+ T cells in vaccinated mice was observed for all the time points of our experiment, reaching the peak at day 30 after infection (Figure 14A). Of note, lung IL-17-producing CD4+ T cells did not coexpress IFN-γ (data not shown). Our group has recently described a novel multifunctional CD4+ T cell population co-expressing IL-17, TNF and IL-2 that is associated with BCG-induced protection for infection with H37Rv (A. Cruz *et al.*, submitted for publication). Given that, we next analyzed by flow cytometry this multifunctional CD4+ T cell population in the lungs of both groups of 02-171 aerosol infected WT mice. The frequency of lung IL-17+TNF+IL-2+ CD4+ T cells at day 14 post-infection was equal in both vaccinated and unvaccinated mice (Figure 14B). Then, in WT unvaccinated mice the frequency decreased until day 20 post-infection being significantly different from vaccinated mice, which showed a higher frequency (Figure 14B). From here on, the frequency of lung IL-17+TNF+IL-2+ CD4+ T cells were similar in both groups of mice (Figure 14B).

The number of lung IL-17+TNF+IL-2+ CD4+ T cells was significantly higher at the days 14 and 30 post-infection in BCG vaccinated mice than in unvaccinated-ones (Figure 14B). Although without statistical significance, in the remaining time points we observed the same tendency (Figure 14B). IL-17 induces the production of several proinflammatory cytokines, among which of GM-CSF [80, 91]. So, we investigated the expression of GM-CSF in the CD4+ T cell population, by flow cytometry. In line with the increased expression of IL-17-producing CD4+ T cells, we observed that BCG vaccinated WT mice had an overall higher frequency and number of lung GM-CSF-producing CD4+ T cells compared to unvaccinated WT mice (Figure 14C). BCG vaccinated WT mice had a significant increased frequency of GM-CSF-producing CD4+ T cells for all time points tested (Figure 14C). In

both vaccinated and unvaccinated WT mice the frequency and number of lung GM-CSF-producing CD4+ T cells increased from the day 20 after infection, peaked at day 30 of infection, decreasing thereafter (Figure 14C). Despite the same tendency the number of GM-CSF-producing CD4+ T cells at days 20 and 30 after infection in vaccinated WT mice were not significantly different from those obtained for WT unvaccinated mice (Figure 14C).

We concluded that upon aerosol infection with 02-171 Mtb strain, BCG vaccination can induce a higher expression of IL-17- and GM-CSF- producing CD4+ T cells that associate with the vaccine-induced antimycobacterial function (Figures 11A, 14A and 14C). Furthermore, the novel IL-17+TNF+IL-2+ multifunctional subset of CD4+T cells also associates with BCG-induced control of 02-171 Mtb infection in WT mice (Figures 11A and 14B).

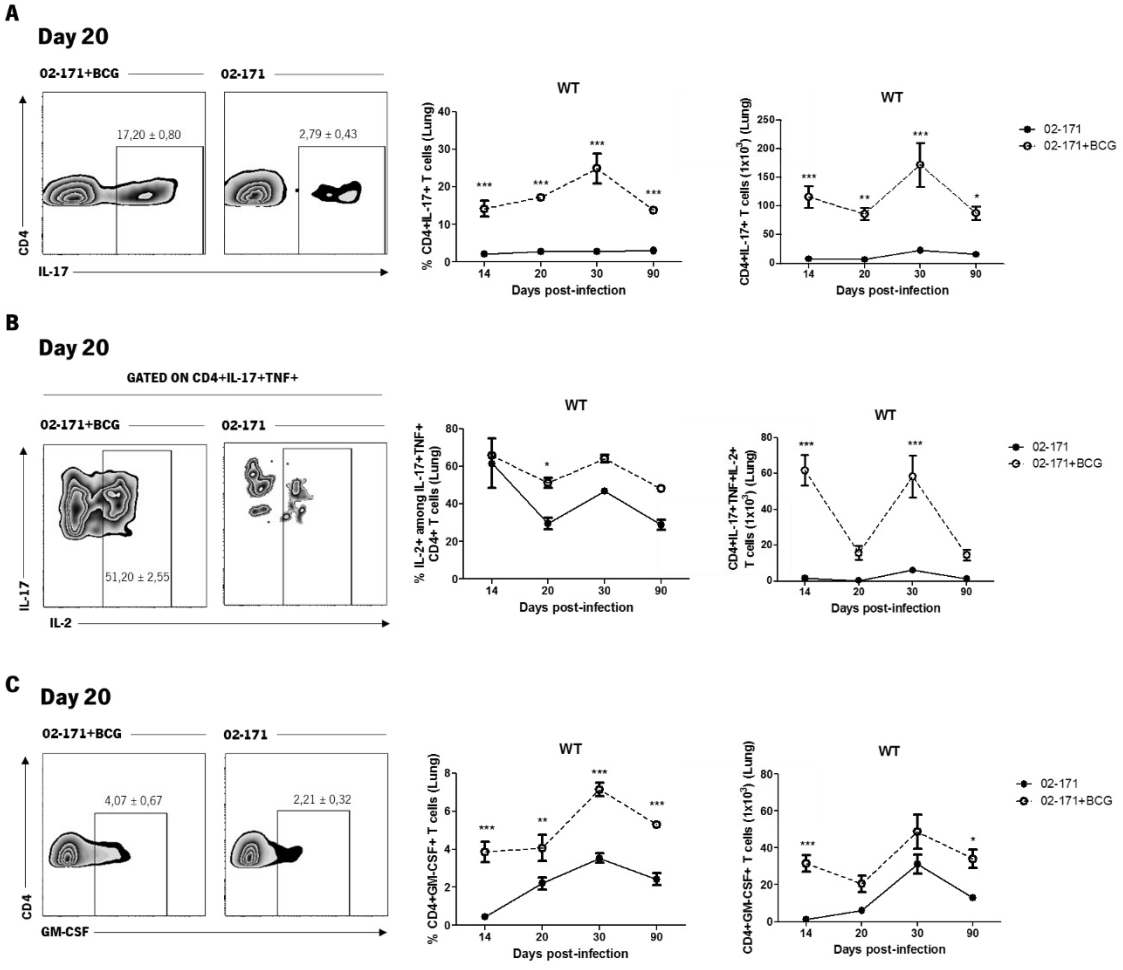


**Figure 14 – BCG vaccination leads to an early accumulation of lung IL-17, GM-CSF and IL-17+TNF+IL-2+ multifunctional CD4+ T cells after Mtb infection.** BCG vaccinated (02-171+BCG) (open circles, dashed line) or unvaccinated (02-171) (closed circles, solid line) WT mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.250 ± 0.037 (Mean ± SEM for 5 animals). Infected mice were

euthanized at the indicated time points after infection. Lung cell suspensions were prepared and cytokine production were analyzed by flow cytometry. **(A)** Representative FACS profile showing IL-17 expression by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of IL-17 positive cells of each experimental group is indicated (left panel). Percentage and total number of IL-17 positive cells on gated CD4+ T cells (middle and right panels). **(B)** Representative FACS profile showing the expression IL-2+ among IL-17+TNF+ by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of IL-17+TNF+IL-2+ cells of each experimental group was indicated (left panel). Percentage and total number of IL-17+TNF+IL-2+ cells on gated CD4+ T cells (middle and right panels). **(C)** Representative FACS profile showing GM-CSF expression by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of GM-CSF positive cells of each experimental group is indicated (left panel). Percentage and total number of GM-CSF positive cells on gated CD4+ T cells (middle and right panels). The data points represent the Mean ± SEM of five mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$)

## 2.5 BCG vaccination prevents severe lung inflammation induced by 02-171 Mtb infection in WT mice

A hallmark of Mtb infection is the granuloma formation, which has an essential function in host protection against the infection [132]. IL-17 is induced upon Mtb infection and contributes to several steps of the immune response, particularly for the infection-induced granuloma formation [132]. Moreover, IL-17 also impacts neutrophil recruitment which is often associated with lung inflammation [80]. Given that we found a higher expression of this cytokine in BCG WT vaccinated mice (Figure 14A), we next investigated the influence of BCG vaccination on the development of lung inflammation upon infection with 02-171 Mtb strain. For that, we stained with H&E the upper right lobe of the lungs of BCG vaccinated and unvaccinated aerosol 02-171 infected WT mice. The lungs of both groups of WT 02-171-infected mice developed progressive lung lesions after day 20 post-infection (Figure 15). Mice that were vaccinated with BCG showed reduced lung inflammation at days 30 and 90 post-infection compared with unvaccinated WT mice (Figure 15). At day 90 post-infection in unvaccinated WT mice, a vast proportion of the lung tissue was occupied with inflammatory infiltrates (Figure 15). As expected, uninfected vaccinated and unvaccinated WT mice did not exhibited lung inflammation (Figure 15).

Although we observed a higher induction of IL-17 production by lung CD4+ T cells in BCG WT vaccinated mice (Figure 14A), this increased expression did not cause lung tissue damage (Figure 15).

Overall, in WT vaccinated mice the inflammatory infiltrates were smaller, less extensive and appeared more organized than those exhibited by unvaccinated WT mice (Figure 15), indicating that BCG-induced immunity controls immunopathology after infection with 02-171 Mtb strain.

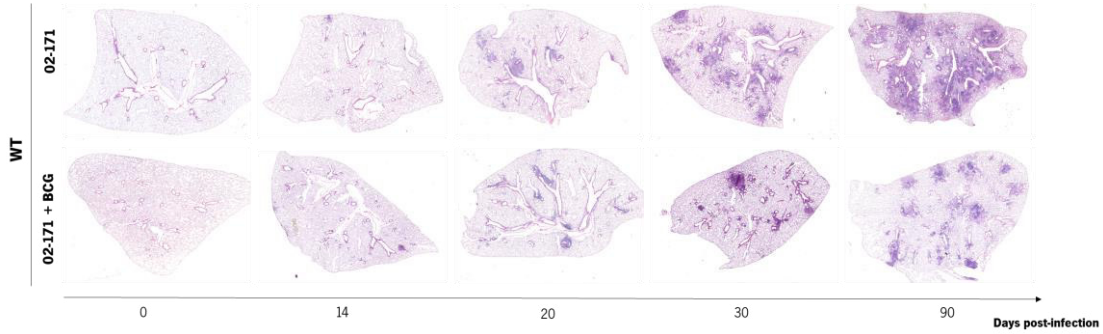


**Figure 15 – Reduced lung inflammation in WT BCG vaccinated mice following aerosol infection with 02-171 Mtb strain.** BCG vaccinated (02-171+BCG) or unvaccinated (02-171) WT mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection ($Log_{10}$ (CFUs) 2.250 ± 0.037 (Mean ± SEM for 5 animals). To assess lung inflammation upper right lobes of the lung were excised from WT unvaccinated (top panel) or WT vaccinated (bottom panel) mice and fixed lung sections were stained with H&E. Representative images from each experimental group were obtained. Images represented for day 0 correspond to uninfected animals. Data are from one experiment.

## 2.6 BCG vaccination protects TLR4 -/- mice against infection by the Beijing TLR4-activating Mtb strain 02-171

Our data add to the controversy regarding the protection conferred by BCG against Mtb strains of the Beijing lineage. Indeed, we clearly show that not only BCG protects mice from infection with 02-171, but also the mechanisms underlying such protection are similar to those in place using Mtb H37Rv, the laboratory reference strain of the Euro-American lineage.

We have recently shown that Mtb strain 02-171 triggers TLR4 in addition to TLR2 [34]. Furthermore, TLR4 agonists have been used as adjuvants in human and also in experimental vaccines, to boost T cell-mediated immune responses [118, 120-122]. Thus, we next investigated the role of TLR4 signaling in the protection conferred by BCG vaccination against 02-171 Mtb infection. For that, TLR4 -/- mice were vaccinated or not with BCG and, 90 days later, infected via the aerosol route with 02-171 Mtb strain.

To assess the effectiveness of BCG vaccination in TLR4 -/- mice, we evaluated the mycobacterial growth in relevant organs namely in the lungs, the primary site of infection, and in the liver, that

represents the dissemination of Mtb. We observed that BCG vaccination, prior to 02-171 infection of TLR4 -/- mice, controlled the bacterial growth in the lungs and in the liver as soon as 14 days post-infection (Figure 16A and B). Indeed, in the lung, BCG vaccination arrested Mtb growth from day 14 up to day 90 of infection (Figure 16A). This strongly suggests that the protection conferred by BCG against Mtb 02-171 infection is long lasting.

In the liver, although we observed a significant protection at day 14 post-infection, there was Mtb growth until day 20 of infection after which time point the bacterial load decreased and stabilized (Figure 16B). These findings indicate that activation of TLR4 by Mtb strain 02-171 does not impact the efficacy of BCG vaccination.

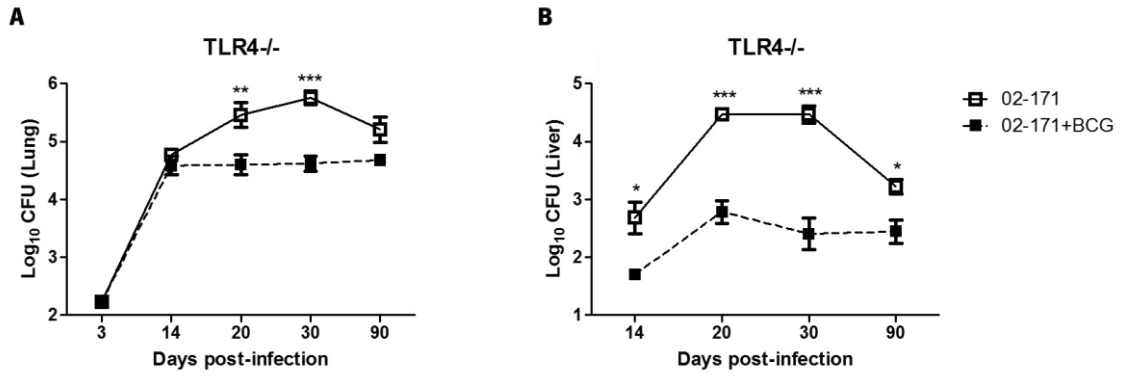


**Figure 16 – TLR4 absence does not influence the effectiveness of BCG vaccination in protecting mice against a Beijing TLR4-activating Mtb strain infection**. BCG vaccinated (02-171+BCG) (closed squares, dashed line) or unvaccinated (02-171) (open squares, solid line) TLR4 -/- mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection. At the indicated time points after infection mice were sacrificed and the bacterial burden determined after 21 days of incubation by plating serial dilutions of lung **(A)** and liver **(B)** homogenates. The initial infection dose into the lungs was $Log_{10}$(CFUs) 2.229±0.056 (Mean ± SEM for 2 animals). The data points represent the Mean ± SEM of at least 4 mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05; **, p≤0.01; ***, p≤0.001)

## 2.7 BCG-induced protection associates with an anticipation of CD4+ T cell response in the lungs of TLR4 -/- mice

We next compared by flow cytometry the CD4+ T cell response in the lungs of vaccinated and unvaccinated TLR4 -/- mice after aerosol challenge with 02-171 Mtb strain. We found that at day 14 of Mtb infection the frequency of lung CD4+ T cells was similar in both vaccinated and unvaccinated TLR4 -/- mice (Figure 17B). Then, the CD4+ T cells in TLR4 -/- vaccinated mice

increased, peaking at day 20 and decreasing afterwards, while in TLR4 -/- unvaccinated mice the maximum percentage of CD4+ T cells was achieved later, at day 30 post-infection (Figures 17A and B). The same trend was observed in terms of CD4+ T cell numbers in the lungs of infected mice (Figure 17B). In all, we observed an anticipation of the peak of CD4+ T cells, which most likely accounts for the early Mtb growth arrest, in TLR4 -/- vaccinated mice (Figure 16A and 17B).

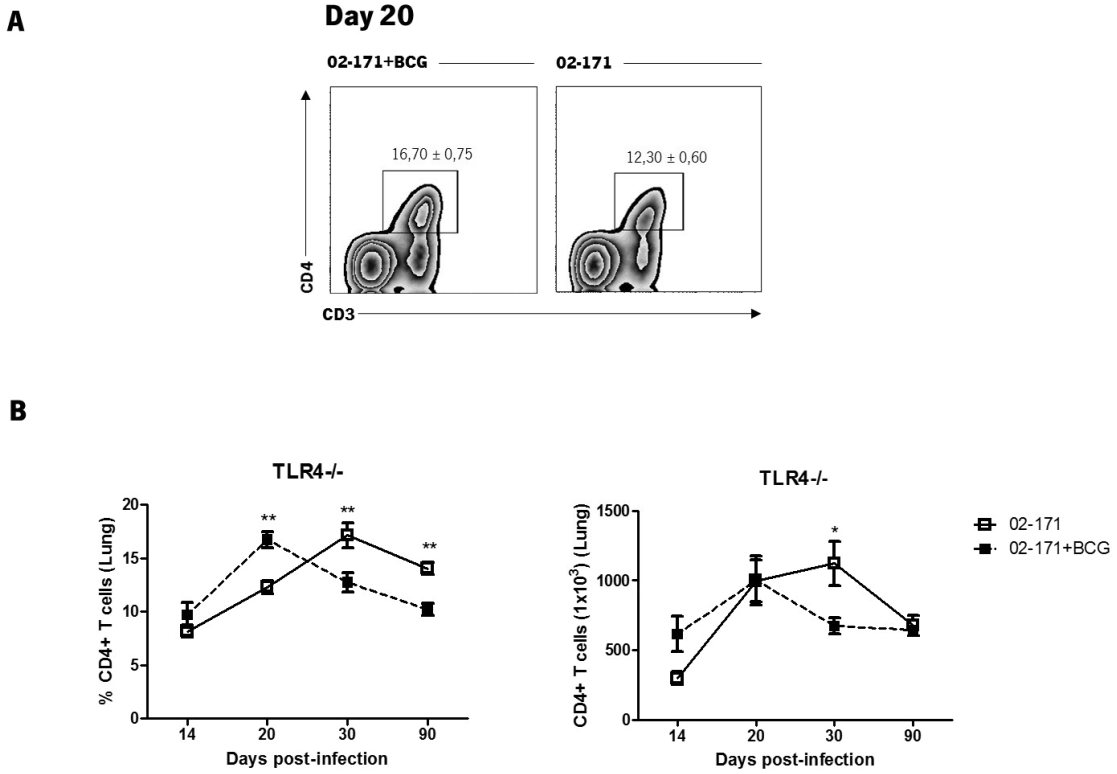


**Figure 17 – BCG-induced protection associates with an increased frequency of lung CD4+ T cell response at day 20 of infection in TLR4-/- mice.** BCG vaccinated (02-171+BCG) (closed squares, dashed line) or unvaccinated (02-171) (open squares, solid line) TLR4 -/- mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection (Log10 (CFUs) 2.229 ± 0.056 (Mean ± SEM for 2 animals). Infected mice were euthanized at the indicated time points after infection. Lung cell suspensions were prepared and the CD4+ T cells were analyzed by flow cytometry. **(A)** Representative FACS profile showing CD4 expression by lung cells at day 20 post-infection. The Mean ± SEM of CD4 positive cells of each experimental group is indicated. **(B)** Percentage and total number of CD4 positive cells in the lung. The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are representative of one experiment. (*, p≤0.05; **, p≤0.01)

## 2.8 BCG-mediated control of 02-171 Mtb infection associates with an early accumulation of IFN-γ and IFN-γ+TNF+IL-2+ multifunctional CD4+ T cell response in the lungs of TLR4 -/- mice

We next compared by flow cytometry the IFN-γ T cell response in the lungs of vaccinated and unvaccinated TLR4 -/- mice after aerosol challenge with 02-171 Mtb strain. TLR4 -/- BCG vaccinated mice exhibited a higher frequency of IFN-γ-producing CD4+ T cells in the lungs on days 14 and 20 after infection compared with TLR4 -/- unvaccinated 02-171-infected mice (Figures 18A and B). Moreover, in TLR4 -/- vaccinated mice we observed an anticipation in the peak of lung IFN-γ-producing CD4+ T cells to day 20 post-infection versus day 30 in unvaccinated mice (Figure 18B). The frequency of this population was similar in both vaccinated and unvaccinated TLR4 -/- mice at days 30 and 90 after Mtb infection (Figure 18B). The same trend was observed for the number of IFN-γ CD4+ T cells (Figure 18B).

Owing to the relevance of IFN-γ-producing multifunctional CD4+ T cells in vaccine-induced protection [130, 131], we next analyzed whether TLR4 signaling during Mtb infection altered the co-expression of IFN-γ, TNF and IL-2 in lung CD4+ T cells by flow cytometry. The frequency of lung IFN-γ+TNF+IL-2+ CD4+ T cells decreased over the time and curiously we observed an increased frequency of this multifunctional CD4+ T cell population in unvaccinated TLR4 -/- mice at day 20 after Mtb infection compared with TLR4 -/- vaccinated mice (Figures 18C and D). The number of lung IFN-γ+TNF+IL-2+ CD4+ T cells in TLR4 -/- vaccinated mice peaked earlier and was significantly higher than that observed in unvaccinated TLR4 -/- mice (Figure 18D). After day 20 of infection the number of this multifunctional CD4+ T cell population in vaccinated mice dropped, becoming similar what was observed for unvaccinated mice (Figure 18D). In accordance with the dynamics observed for the IFN-γ T cell response, we also observed an early induction and accumulation of this multifunctional T cell population in vaccinated mice (Figure 18D) that might account for the better control of 02-171 Mtb infection in TLR4 -/- BCG vaccinated mice compared with TLR4 -/- unvaccinated-ones.

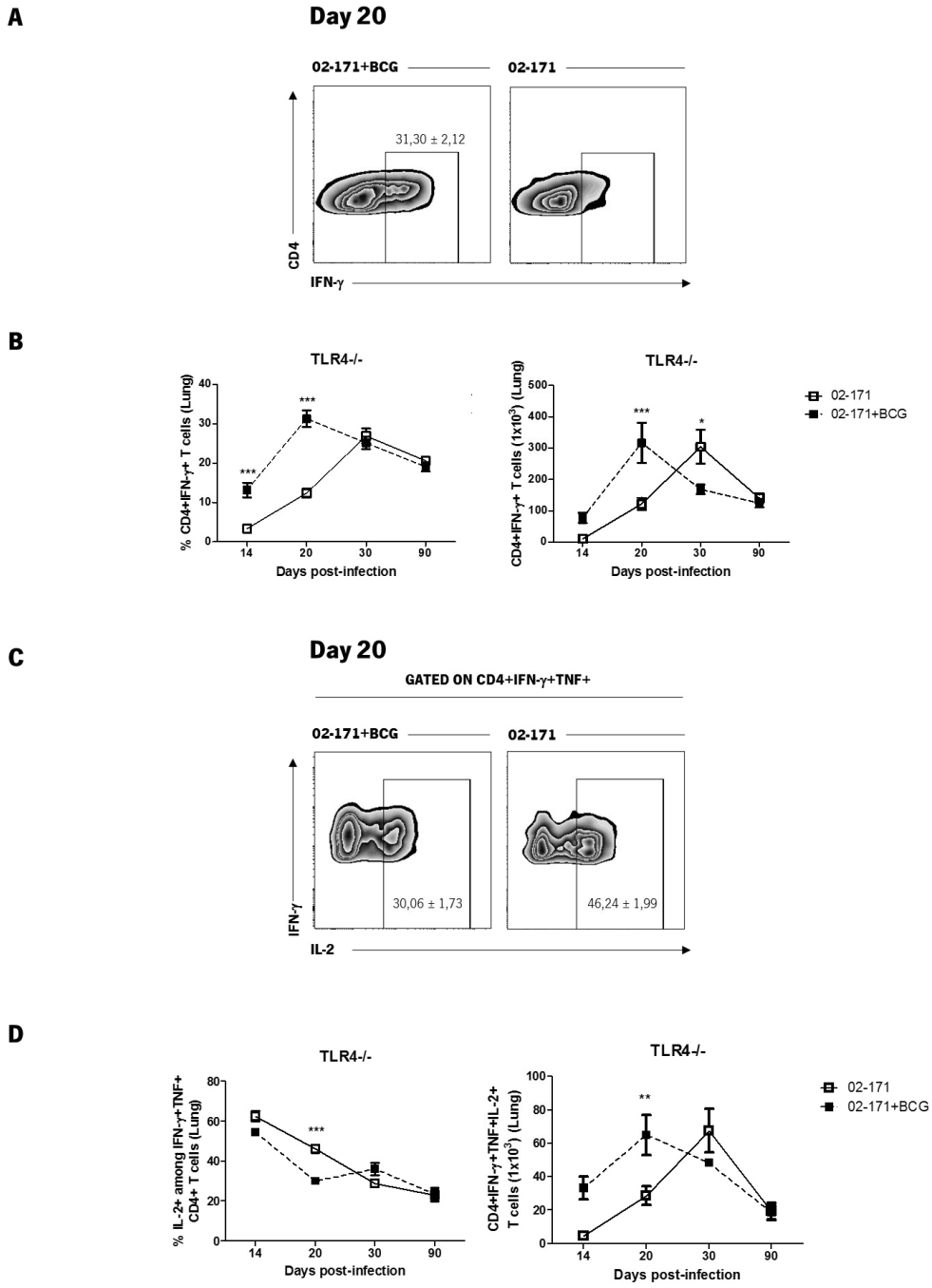


**Figure 18 – BCG vaccination leads to an early accumulation of lung IFN-γ+TNF+IL-2+ multifunctional CD4+ T cells in the lungs of TLR4-/- mice.** BCG vaccinated (02-171+BCG) (closed squares, dashed line) or unvaccinated (02-171) (open squares, solid line) TLR4-/- mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection (Log10 (CFUs) 2.229 ± 0.056 (Mean ± SEM for 2 animals). Infected mice were euthanized at the indicated time points after infection. Lung cell suspensions were prepared and cytokine production was analyzed by flow cytometry. **(A)** Representative FACS profile showing IFN-γ expression by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of IFN-γ positive cells of each experimental group is indicated. **(B)** Percentage and total number of IFN-γ positive cells on gated CD4+ T cells. **(C)** Representative FACS profile showing the expression of IL-2+ among IFN-γ+TNF+ by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of IFN-γ+TNF+IL-2+ cells

of each experimental group is indicated. **(D)** Percentage and total number of IFN-γ+TNF+IL-2+ on gated CD4+ T cells. The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, p≤0.05; **, p≤0.01; ***, p≤0.001)

## 2.9 BCG-mediated control of 02-171 Mtb infection associates with an increased induction of GM-CSF but not IL-17 or IL-17+TNF+IL-2+ CD4+ T cell responses in the lungs of TLR4 -/- mice

We next investigated by flow cytometry the IL-17 CD4+ T cell response in the lungs of vaccinated and unvaccinated TLR4 -/- mice after 02-171 aerosol challenge. In contrast to what we observed for WT BCG vaccinated mice (Figure 14A), we found that BCG immunization did not induce a higher frequency and number of IL-17-producing CD4+ T cells in the lungs of TLR4 -/- mice (Figure 19A). At days 14, 20 and 90 after Mtb infection the number of IL-17-producing CD4+ T cells was indeed similar among vaccinated and unvaccinated TLR4 -/- mice (Figure 19A). In TLR4 -/- BCG vaccinated mice the maximum number of IL-17 CD4+ T cells occurred on day 20 after infection, whereas for TLR4 -/- unvaccinated animals the number of IL-17-producing CD4+ T cells continued to increase until day 30 of infection, dropping afterwards (Figure 19A). The frequency of those cells were identical for both groups of TLR4 -/- mice for all the time points tested (Figure 19A).

We next analyzed by flow cytometry the co-expression of IL-17, TNF and IL-2 by CD4+ T cell population in the lungs of vaccinated and unvaccinated TLR4 -/- 02-171-infected mice. The frequency of lung IL-17+TNF+IL-2+ CD4+ T cells was similar for both groups of mice at day 14 of infection (Figure 19B). In TLR4 -/- unvaccinated mice, the frequency of this multifunctional CD4+ T cell population increased until day 20 post-infection and stabilized afterwards, being significantly higher than in vaccinated mice at the days 20, 30 and 90 post-infection (Figure 19B). In what concerns cell numbers, this T cell population peaked in TLR4 -/- unvaccinated mice at day 30 of infection (Figure 19B). Only at this time point was the number of IL-17+TNF+IL-2+ CD4+ T cells significantly higher than in TLR4 -/- vaccinated animals (Figure 19B). In fact, in vaccinated TLR4 -/- mice the number of IL-17+TNF+IL-2+ CD4+ T cells remained relatively stable for all time points tested (Figure 19B). Overall, our data suggest that TLR4 triggering is important for the induction of IL-17-producing CD4+ T cells during BCG vaccination. However, whether this signal is required during vaccination, infection or both, remains to be elucidated.

Finally, we analyzed the production of GM-CSF among the CD4+ T cell population. BCG vaccinated TLR4 -/- mice showed a significantly higher frequency and number of lung GM-CSF-producing

CD4+ T cells than unvaccinated mice at day 20 post-infection (Figure 19C). Although not statistically different, at day 14 of infection the same trend was observed (Figure 19C). For BCG vaccinated TLR4 -/- mice, the frequency and number of GM-CSF-producing CD4+ T cells peaked at day 20 of infection, decreasing thereafter (Figure 19C). This represented an anticipation of this T cell population, as for unvaccinated TLR4 -/- mice, the peak of GM-CSF-producing CD4+ T cells happened on day 30 post-infection (Figure 19C). Additionally, we observed that for TLR4 -/- mice the increased production of GM-CSF by CD4+ T cells was not associated with an increased IL-17 CD4+ T cell response (Figures 19A and C) as we hypothesized for WT mice.

As for WT mice, the data suggest that BCG vaccination can induce an early expression of GM-CSF-producing CD4+ T cells upon aerosol infection with 02-171 Mtb strain that might associate with the vaccine-induced antimycobacterial function. The differentiation of these cells appears therefore to be independent of TLR4.

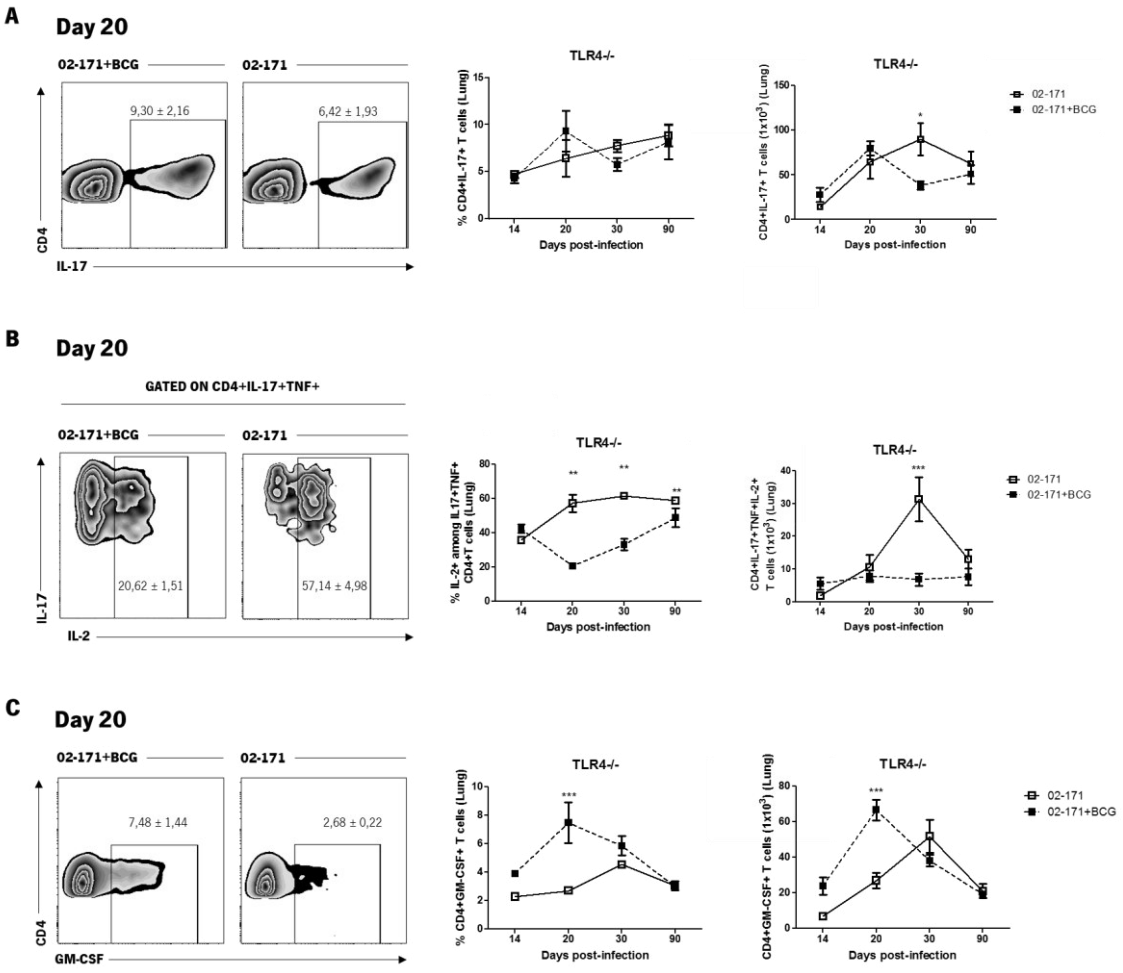

**Figure 19 – BCG vaccination leads to an early accumulation of lung GM-CSF but not IL-17 and IL-17+TNF+IL-2+ multifunctional CD4+ T cells after Mtb infection of TLR4 -/- mice.** BCG vaccinated (02-171+BCG) (closed squares, dashed line) or unvaccinated (02-171) (open squares, solid line) TLR4-/- mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection (Log10 (CFUs) 2.229 ± 0.056 (Mean ± SEM for 2 animals). Infected mice were euthanized at the indicated time points after infection. Lung cell suspensions were prepared and cytokine production was analyzed by flow cytometry. **(A)** Representative FACS profile showing IL-17 expression by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of IL-17 positive cells of each experimental group is indicated (left panel). Percentage and total number of IL-17 positive cells on gated CD4+ T cells (middle and right panels). **(B)** Representative FACS profile showing the expression of IL-2+ among IL-17+TNF+ by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of IL-17+TNF+IL-2+ positive cells of each experimental group is indicated (left panel). Percentage and total number of IL-17+TNF+IL-2+ positive cells on gated CD4+ T cells (middle and right panels). **(C)** Representative FACS profile showing GM-CSF expression by lung CD4+ T cells at day 20 post-infection. The Mean ± SEM of GM-CSF positive cells of each experimental group is indicated (left panel). Percentage and total number of GM-CSF positive cells on gated CD4+ T cells (middle and right panels). The data points represent the Mean ± SEM of at least four mice per group and the statistical significance was determined by Two-way ANOVA with Bonferroni post-test. Data are from one experiment. (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$)

## 2.10 BCG immunization reduces immunopathology after 02-171 Mtb infection in TLR4 -/- mice

We next evaluated the lung inflammation after 02-171 Mtb infection of BCG vaccinated and unvaccinated TLR4 -/- mice. For that, we stained with H&E the upper right lobe of the lungs of both groups of TLR4 -/- mice. The lungs of both, vaccinated and unvaccinated, TLR4 -/- 02-171-infected mice exhibited progressive lung lesions after day 20 post-infection (Figure 20). Mice that were vaccinated with BCG showed reduced lung inflammation at days 30 and 90 post-infection compared with unvaccinated TLR4 -/- mice (Figure 20). Overall, we observed a slower progression of lung inflammation in BCG vaccinated TLR4 -/- mice compared to unvaccinated mice (Figure 20). Furthermore, it seems that at day 90 of Mtb infection both vaccinated and unvaccinated TLR4 -/- mice had a reduced lung inflammation compared with the respective groups of WT animals (Figures 15 and 20).

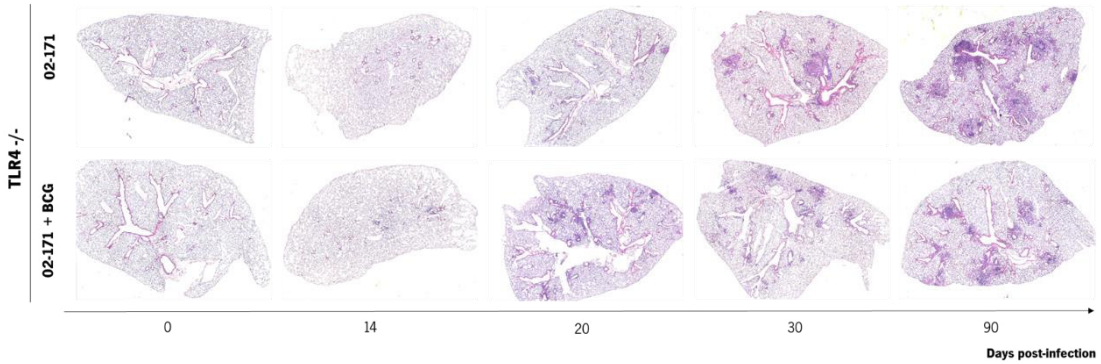


**Figure 20 – BCG immunization reduces lung inflammation in TLR4 -/- mice infected with 02-171 Mtb strain.** BCG vaccinated (02-171+BCG) or unvaccinated (02-171) TLR4 -/- mice were infected with Mtb strain 02-171 via the aerosol route with a low dose of infection (Log10 (CFUs) 2.229 ± 0.056 (Mean ± SEM for 2 animals). To assess lung inflammation upper right lobes of the lung were excised from TLR4 -/- unvaccinated (top panel) or TLR4 -/- vaccinated (bottom panel) mice and fixed lung sections were stained with H&E. Representative images from each experimental group were obtained. Images represented for day 0 correspond to uninfected animals. Data are from one experiment.

# DISCUSSION

TB still represents a severe public health problem [1]. Although anti-TB drugs are available for decades, Mtb still causes the death of nearly two million people annually [1]. Currently, the co-infection with HIV and the emergence of MDR strains, related to noncompliance or to an inappropriate drug regimen, have further complicated the TB control [1, 2]. BCG vaccination has demonstrated its efficacy in preventing childhood TB, but the limited and variable protection against adulthood TB leaves this serious public health problem without a widely efficacious vaccine [101, 102, 133].

One of the most intriguing issues in TB is the large spectrum of TB outcomes, which can range from clearance, to latent infection to active disease affecting multiple organs – miliary TB [7]. In response to Mtb infection, the immune response is activated, but such activation needs to be cautiously balanced [56, 99]. On one hand, the immune response has to be strong enough to fight the infection. On the other hand, it needs to be controlled in order to prevent extensive tissue damage [56, 99]. However, in most of cases of Mtb infection, the immune response generated by the host is not efficient enough to clear the pathogen, but instead it leads to an inactivated form of the disease [56, 99]. When the fine balance established during latency is broken, Mtb infection rapidly reactivates, which happens in 5 to 10% of Mtb cases, and progresses to active disease [6, 56, 99].

Both host and pathogen features can contribute to the development of active TB [6, 7, 15]. Among them, the pathogen strain variability, which could account for differences in the virulence [12, 21]. The human genetic background, singular polymorphisms, life habits and comorbidities could also associate with increased TB susceptibility [15, 43, 86].

Studies in animals models using clinical isolates showed the complexity and heterogeneity of the immune response induced in the host [21, 134, 135]. These observations demonstrate some of the limitations in the exclusive use of laboratory strains, which can also oversimplify this complex disease. Therefore, the use of clinical isolates of Mtb could be one way to overcome this issue, given that they are likely less adapted to laboratory conditions, thus better reflecting the progress of Mtb strains during infection. Moreover, TB vaccines are normally tested against the laboratory strains and limited information is available to understand whether BCG vaccine will be effective against the newly emerging clinical strains of Mtb, as Beijing strains [20]. This is an important issue to take into account, because the wide diversity in the virulence and pathogenesis of Mtb strains could have a huge impact on the diagnosis, treatment response and also BCG efficacy [136].

The emergence and spread of new Mtb strains have raised considerable concern. Of particular significance is the Beijing family of strains that is believed to have increased pathogenicity, a propensity to evade the protection mediated by BCG and increased drug resistance [18, 19, 21]. It has been described that human macrophages have a differential cytokine production after infection with different Mtb strains therefore showing a distinct interaction with innate immune cells and a heterogeneous response [135]. Our recent data showed that in a panel of Beijing strains, most of the strains were recognized by TLR2 in BMDM. However, 02-171 Mtb strain was also recognized by TLR4 [34]. Therefore, we suggest that differential TLR recognition of Mtb strains may be one of the host molecular basis underlying heterogeneous immune responses to Mtb.

In the Chapter I of this thesis we investigated the role of TLR4 activation on T cell development during primary infection with 02-171, a Beijing TLR4-activating Mtb strain. For that, we infected WT and TLR4 -/- mice via the aerosol route with a low dose of 02-171 Mtb strain.

At day 25 post primary infection with 02-171 Mtb strain, we observed that TLR4 triggering increased the frequency of CD4+ T cells. However, when we looked to the total number of CD4+ T cells no difference was found. The total number of lung cells in both groups of mice was similar at this time point (data not shown). The number of CD4+ T cells in WT and TLR4 -/- animals, did not reach significance may be due to some heterogeneity within the groups.

We hypothesized that the increment in the frequency of CD4+ T cell can be explained by differential local proliferation in the infected lungs. To test our hypothesis, 24 hours before the experimental time point, both strains of 02-171-infected mice were intraperitoneally injected with BrdU, and cells actively synthesizing DNA were detected by flow cytometry. We found that the increased frequency of CD4+ T cells in WT mice was independent of local proliferation. Therefore, it most likely is a consequence of enhanced cell recruitment to the lungs. In support of this hypothesis is the finding that the expression of *Cxcl10* and *Cxcl11*, chemokines involved in the recruitment of T cells, was increased in WT mice as compared to TLR4 -/- at day 20 post-infection. Other hypotheses that could explained the difference observed are the increased survival in the lungs or the earlier priming in the LN of CD4+ T cells in infected WT mice compared with TLR4 -/-. These hypotheses need further clarification and are the scope of ongoing studies.

Furthermore, TLR4 activation seems not to influence the phenotype of CD4+ T cells at least for the activation markers tested.

We next investigated the impact of TLR4 activation during 02-171 Mtb infection on the lung IFN-γ response. We found that TLR4 triggering did not impact lung IFN-γ production by CD4+ T cells. However, when we looked for *Ifng* expression in the total lung we observed that at day 32 of infection TLR4 -/- mice showed significant higher levels of this cytokine when compared to WT animals. CD4+ T cells are the primary source of IFN-γ during the adaptive immune response to Mtb infection [56]. However, other cell types such as CD8+ T cells, NK cells and NKT cells can also produce IFN-γ upon Mtb challenge [56, 137]. It is therefore possible that these other cell populations contribute for the higher IFN-γ expression in the infected lungs of TLR4 -/- mice, but such hypothesis needs further confirmation. Furthermore, the increased IFN-γ production in TLR4 -/- does not associate with a better control of Mtb infection, further supporting the idea that IFN-γ, by itself, is not sufficient to induce Mtb growth arrest [108].

Additionally, we have to keep in mind that the production of cytokines by CD4+ T cells was assessed after *ex vivo* re-stimulation using PMA and ionomycin together with brefeldin A. This method will lead to the activation of several intracellular signaling pathways that allows us to identify what is the cytokine profile of CD4+ T cells in the infected lungs [138]. *Ex vivo* re-stimulation reveals a specific phenotype that T cells have acquired during infection, but that may not necessarily be "active" at the site of infection.

We next investigated the kinetics of lung IL-17-producing CD4+ T cells by flow cytometry. We observed that the frequency of IL-17-producing CD4+ T cells was enhanced in TLR4 -/- mice as compared to WT 02-171-infected animals. Indeed, the dynamics of IL-17 CD4+ T cell response in TLR4 -/- seemed anticipated as compared with WT mice. Nevertheless, the difference observed for IL-17 CD4+ T cells did not impact the control of 02-171 Mtb infection, with the TLR4 -/- mice being as resistant as WT-infected mice. Indeed, it has been reported that IL-17 does not play a major protective role in primary Mtb infection, being mainly involved in the maintenance of the inflammatory response [139, 140].

When we investigated the relative mRNA expression of *Il-17* in the total lung we did not observe significant differences among WT and TLR4 -/- mice. Thus, it is possible that in the total lung the contribution of other cell types is masking the differences observed in CD4+ T cells. Indeed, it has been described that upon Mtb infection, γδ T cells rather than CD4+ T cells are an important source of IL-17 [69]. In a future approach, we will include in our FACS staining a specific marker to evaluate the production of IL-17 by γδ T cells. In addition, we could also perform an enrichment of lung CD4+ T cells by positive selection to evaluate the *Il-17* mRNA expression. Moreover, we

could re-stimulate lung cells with PMA plus ionomycin without brefeldin A to measure the IL-17 protein in the lung supernatant by ELISA. One another hand, it is possible that the higher production of IL-17 by lung CD4+ T cells was not reflected at the mRNA expression level.

On possible explanation for the increased production of IL-17 in TLR4 -/- infected mice compared to WT mice is that TLR4 in addition to MyD88 also trigger an alternative signaling pathway via TRIF adaptor molecule, that allow the induction of a different profile of the immune response, specifically the type I IFN production [23, 24]. Type I IFN has been shown to negatively impact the generation of Th17 cells, by inducing IL-27 production, which has been reported as a negative regulator of IL-17 secretion [141]. However, this is a conflicting issue because other reports state that TLR4 and dectin1 receptors are responsible for Mtb-induced IL-17 responses [140]. Accordingly to the last work we expected to find lower IL-17 production in TLR4 -/- than in WT mice. However, this was not the case, therefore it is probable that dectin1 and possible other PRRs compensate for the IL-17 production. Therefore, in the future it will be interesting understand which are the molecular mechanisms responsible for IL-17 inducing upon Mtb infection and the possible cross-talk between PRRs.

Given the role of IL-17 in the maintenance of the inflammatory response after Mtb infection [139], and despite the differences observed in the IL-17 response. We did not observe significant differences in the number of neutrophils neither in lung inflammation among WT and TLR4 -/- infected mice. We also concluded that TLR4 activation by 02-171 Mtb strain did not impact *Nos2* expression and NOS2 protein, since we observed similar levels in both groups of Mtb-infected mice. During Mtb infection, IFN-γ and TNF signaling synergize to the induction of NOS2 [5, 56]. Our previous work showed that the absence of TLR4 triggering in 02-171 intranasal infected mice decreased the cytokine expression, such as IFN-γ and TNF, which is accompanied by a decreased *Nos2* expression with consequence lack of protection against 02-171 Mtb infection. In our work, we did not observe significantly differences in IFN-γ and TNF production, neither *Nos2* expression in the presence or not of TLR4 triggering. Therefore, we hypothesized that the levels of IFN-γ and TNF can at least partially explain *Nos2* expression. Reasons for our apparent contrasting data are discussed below.

In accordance with what we observed for the IFN-γ response, lung pathology and NOS2 expression the progression of bacterial burden in the lungs and in the LN were similar for both strains of mice at all time points tested. It is still possible that TLR4 absence impacts the protection against Mtb in a chronic phase of the infection. Therefore in a future experiment we have to include later time

points as 90 or 150 days post-infection, as there is a precedent in the literature to support this hypothesis. It has been described that TLR2 -/- mice were more susceptible to Mtb infection than control animals [28]. In this reported work, the bacterial burden in the lungs was only measured at two time points, specifically at days 30 and 150 post-infection, and only in the latest time point, differences were observed for both experimental groups [28].

Altogether our data suggest that TLR4 activation during a primary infection by a TLR4-activacting strain, at least in an aerosol standard infection dose and in the time points tested, may not play a significant role in the course of infection. These findings, are in apparent contrast with what we published before, which suggest a protective role for TLR4 triggering upon intranasal infection with 02-171 Mtb strain [34]. These conflicting results, can be explained by the different route of infection (intranasal vs aerosol) and/or by the initial inoculum dose (low vs high dose). In what respects the route of infection, differences between the intranasal and aerosol models have not been explored yet. However, during an aerosol infection the bacteria is delivered to the lower respiratory tract, whereas after an intranasal infection, the bacteria contacts with the upper respiratory tract until it reaches the lower airways, thus interacting with different cells and possibly activating different pathways from both the upper and lower respiratory tract, which could influence the outcome of the Mtb infection. In support of the initial inoculum dose, it has been reported in the literature that, at low dose, TLR2 -/- are as resistant to infection as WT mice, however with a higher infection dose TLR2 -/- mice exhibited an increased susceptibility compared with control animals, suggesting that TLR2 triggering is needed for the protection to a higher Mtb challenge [33]. Moreover, in murine models of B cell deficiency and IL-17 deficiency showed that upon high, but not low inoculum dose of Mtb, mice were more susceptible to infection compared to control mice [72, 73, 80]. Additionally, we have also data from our laboratory that demonstrate that a mild although significant difference in the inoculum size could alter the susceptibility of mice NOS2 -/- to Mtb infection.

Thus, in the attempt to unmask the role of TLR4 triggering on the control of Mtb infection by a TLR4-activating Mtb strain not apparent in the standard aerosol experimental infection (around 100 to 200 CFU), we are presently performing an experiment in which we elevated the initial infection dose of Mtb to approximately 500 CFUs.

In the Chapter II of this thesis we explored the importance of the TLR4 triggering by 02-171 Mtb strain during a recall response after BCG vaccination.

Recently, it has been postulated that the BCG-induced protective immunity may be Mtb strain-specific which may contribute to the limited efficacy of this vaccine [19]. The proposed model is consistent with the growing body of evidence on the genetic heterogeneity and phenotypic differences among Mtb strains [15, 129]. Therefore, differential host-pathogen interactions caused by phenotypic differences among Mtb strains could alter BCG-induced immunity, causing limited protective responses to specific Mtb strains [142].

Among the most prevalent of the Mtb genotypes are the Beijing strains which have been associated with worldwide TB outbreaks [17, 18]. It also been reported that, in mice, BCG vaccination showed less protection against challenge with a Beijing strain than against infection with the H37Rv strain [21]. These data are not definitive as another work reported that in the mouse model of pulmonary Mtb infection, BCG immunization protected equally well against infection with diverse Mtb strains, including strains of the Beijing genotype, thus suggesting that strain-specific resistance to BCG-induced protective immunity may be unusual [115]. Thus, the association between Beijing strains and the efficacy of BCG vaccination is still unclear. Taking into account this problematic and our previous published work on the activation of TLR4 by particular Mtb strains [34], we hypothesized that the differential TLR recognition of Mtb strains could also account for the variability of BCG-induced protection.

Our findings showed that BCG vaccination is effective at controlling the infection caused by 02-171 Mtb strain, suggesting that BCG immunization can be protective against an infection caused by a Beijing Mtb strain that activates TLR4. Our results, thus favor the hypothesis that not all Beijing strains are resistant to protection afforded by BCG. Thus, the idea that BCG vaccination does not protect against Beijing genotypes needs to be revisited. Only a limited number of comparative studies have been performed to examine post-vaccination protection induced by BCG against Beijing strains. Thus it will be interesting to extend our study to a larger panel of Mtb isolates from different lineages to advance our knowledge on whether BCG efficacy depends on the heterogeneity of Mtb strains and whether it associates with differential TLR recognition.

Although BCG protected both WT and TLR4 -/- mice from 02-171 infection, some differences were observed, which may be of interest. Indeed, in vaccinated TLR4 -/- mice the bacterial burden was controlled from day 14 post-infection up to day 90. At day 14 post-infection, although protected, BCG vaccinated WT mice had not yet arrested Mtb growth, with the infection progressing at a slower rate until day 20. From this time point on, the protection was similar for both strains of immunized mice. It will now be interesting to understand why in WT vaccinated mice the Mtb

growth does not stop at day 14 post-infection as we observed for vaccinated TLR4 -/- mice. These results might suggest that in WT vaccinated mice the protective T cell response arrived early to the lungs although, was not sufficient to control the infection. In the liver, the TLR4 triggering did not impact the protection evoked by BCG.

We next investigated the role of TLR4 triggering on the development of CD4+ T cell responses in BCG vaccinated mice. Overall, we observed that WT vaccinated mice compared to unvaccinated-ones showed an earlier IFN-γ CD4+ T cell response that associates with Mtb infection control. Interestingly, in TLR4 -/- mice the highest levels of IFN-γ and multifunctional IFN-γ+TNF+IL-2+ T cells were achieved at day 20, while in TLR4 -/- unvaccinated mice and in both WT vaccinated and unvaccinated mice the same happens later on at day 30 post-infection. These data suggest that the dynamics of CD4+ T cells after BCG vaccination is different in WT and TLR4 -/- animals, but the mechanism underlying this difference remain elusive.

It is becoming increasingly recognized that the quality of vaccine-induced T cell responses is crucial for developing effective immunization strategies [143]. It has been proposed that multifunctional T cells secreting multiple cytokines are able to proliferate upon encounter with antigen and are more likely than single cytokine producers to represent correlates of protective immunity in chronic infections [144]. The factors responsible for the better efficacy of multifunctional T cells are unknown but possibly include the ability of multifunctional T cells to secrete simultaneously high levels of cytokines. IFN-γ and TNF secretion could synergize for intracellular killing, and IL-2 act on promoting further T cell expansion [143].

In humans some reports have suggested that multifunctional Mtb-specific CD4+ T cells play an essential role in protective immunity against TB [145]. Additionally, mouse studies with leishmaniosis and TB vaccines have shown an association between vaccine-induced multifunctional T cells and vaccine-induced protective immunity against intracellular pathogens [108, 111, 130].

However, another study reported that the frequency of multifunctional CD4+ T cells producing several cytokines (IFN-γ, TNF and IL-2) is significantly increased in individuals with active TB disease, not supporting the existing idea that such responses might be correlated with protection [114].

In our studies, multifunctional T cells are also presented and seem to associate with vaccine-induced protection, so their importance comes up as robust. Clearly, further characterization of

multifunctional T cell responses induced in both mice and humans is needed to assess whether these populations are actually conferring protection against Mtb infection.

It has been shown that IL-17-producing cells mediate vaccine-induced protective immunity by promoting an earlier recruitment of IFN-γ-producing cells to the Mtb-infected lungs [103].

In WT mice, BCG vaccination, induces a higher frequency and number of lung IL-17 producing CD4+ T cells. In contrast, in TLR4 -/- mice, BCG immunization, does not impact the IL-17 CD4+ T cell response, and both vaccinated and unvaccinated TLR4 -/- -infected mice exhibited similar levels of IL-17+ CD4+ T cells. As explained above, vaccination induces a population of 17-producing cells that facilitate the influx of memory protective effector T cells. If so, then this does not explain the similar BCG-induced protection in TLR4 -/- BCG-vaccinated mice compared to WT BCG-vaccinated animals, in which the lung IL-17 CD4+ T cell response was not potentiated by vaccination. Thus, our results suggest that having lower levels of IL-17 production may not impact the BCG-induced protection. It will be interesting to investigate whether in TLR4 -/- mice in response to BCG, other mechanisms can compensate the lack of vaccine-induced IL-17, or alternatively if the increased production of IL-17 in TLR4 -/- during primary infection with 02-171 Mb infection was sufficient and BCG could not improve such response. Indeed, robust IL-17 responses do not necessarily associate with better protection, because boosting BCG vaccinated mice with MVA85A did not increase the protective efficacy of the BCG vaccine against Mtb, despite dramatically increased IL-17 and IFN-γ responses [146]. Additionally, repeated BCG vaccination of Mtb infected mice also results in increased IL-17 responses, neutrophil recruitment and consequently lung tissue damage [94].

We next evaluated the lung inflammation in both strains of mice. We found that BCG vaccination reduced the formation of severe inflammatory foci induced by 02-171 Mtb strain in both WT and TLR4 -/- mice. At day 90 post-infection, unvaccinated TLR4 -/- mice appear to exhibit a reduced lung inflammation as compared with unvaccinated WT animals. This is in line with, BCG-induced IL-17 production was not associated with increased lung inflammation, being rather associated with immunopathology control. Thus, we hypothesized that vaccine-induced IL-17 production might be related with control of lung tissue damage. Indeed, we found that unvaccinated 02-171-infected TLR4 -/- mice showed a higher production of IL-17 compared with unvaccinated WT mice, which is correlated with reduced lung inflammation in TLR4 -/- unvaccinated mice compared to WT unvaccinated-ones. The increased IL-17 production in vaccinated WT mice also correlates with reduced lung inflammation. Indeed, vaccinated TLR4 -/- mice, in which IL-17 was not further

induced by BCG, the improvement in lung inflammation induced by BCG, does not seem so pronounced. Furthermore, a recent study showed that in mice IL-17 may induce disease-associated tissue inflammation via TLR4 signaling [147]. In this study IL-17 was overexpressed through adenovirus-mediated gene transfer in TLR4 -/- mice, and the authors found an absence of neutrophil accumulation, and lack of expression of proinflammatory cytokines downstream of TLR4 in multiple tissues, suggesting that TLR4 may act downstream of IL-17 [147]. This is in accordance with our results because in TLR4 -/- unvaccinated mice, compared to WT unvaccinated-ones, although we had more IL-17, we observed less lung inflammation, suggesting that TLR4 triggering may be downstream IL-17-induced inflammation. However, these issues need further investigation. In a future approach, it will be important to perform a morphometric analysis to score lung inflammation, in a more accurate way.

The idea that IFN-γ is needed but not sufficient for Mtb control is supported by several studies using deficient mouse models [116]. For instance, GM-CSF deficient mice succumbed quickly following Mtb infection [148]. As these mice produce IFN-γ, their failure to limit Mtb infection point out that other important pathways are essential for protective immunity against Mtb infection [116]. It has been reported that GM-CSF production could contribute to host resistance against TB [116]. Indeed, the presence of GM-CSF-specific autoantibodies that block GM-CSF functionality have been associated with pulmonary TB in healthy people [116]. Our results further support this idea, since both WT and TLR4 -/- vaccinated mice showed increased frequency and number of GM-CSF-producing CD4+ T cells. Thus, other immune factors beyond IFN-γ and IL-17, such as GM-CSF needs to be considered as a possible important player in vaccination.

Further investigation is needed in order to identify other immunological functions that protect against Mtb infection and to define the T cell responses that must be induced to optimize vaccine efficiency. Furthermore, such investigations will certainly contribute to define and validate biomarkers of protective immunity against Mtb infection, that gain increased importance with the appearance of novel TB vaccine candidates.

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